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CONTENTS OF THIS ISSUE

Isonicotinyl Hydrazones as Antitubercular Agents and Derivatives for Identification of Aldehydes and Ketones 513
Peter P. T. Sah and *S. Anderson Peoples*

Investigation of Some Complexes Formed in Solution by Caffeine. V. Interactions Between Caffeine and *p*-Aminobenzoic Acid, *m*-Hydroxybenzoic Acid, Picric Acid, *o*-Phthalic Acid, Suberic Acid, and Valeric Acid 524
Takeru Higuchi and *John L. Lach*

Investigation of Complexes Formed in Solution by Caffeine. VI. Comparison of Complexing Behaviors of Methylated Xanthines with *p*-Aminobenzoic Acid, Salicylic Acid, Acetylsalicylic Acid, and *p*-Hydroxybenzoic Acid 527
Takeru Higuchi and *John L. Lach*

The Kinetics of Degradation of Chloramphenicol in Solution. III. The Nature, Specific Hydrogen Ion Catalysis, and Temperature Dependencies of the Degradative Reactions 530
Takeru Higuchi and *Arnold D. Marcus*

Studies on the Stability of Fumagillin. II. Photolytic Degradation of Crystalline Fumagillin 536
Thomas E. Eble and *Edward R. Garrett*

Studies on the Stability of Fumagillin. III. Thermal Degradation in the Presence and Absence of Air 539
Edward R. Garrett

The *in Vitro* Evaluation of the Antibacterial Activity of Undi Oil (*Calophyllum inophyllum* Linn.) 543
S. G. Bhat, J. G. Kane, and *A. Sreenivasan*

Some Attempts to Prolong the Duration of Action of 2,2-Diethyl-1,3-Propanediol (Prenderol®) 547
Irwin H. Slater, John F. O'Leary, and *E. J. Pribyl*

The Electrolytic Effects of Licorice Preparations in the Adrenalectomized Rat 551
William E. Hassan, Jr., Joseph F. Palumbo, and *Fred Elmadjian*

The Effect of Freeze-Drying on Chlorophyll in the Leaves of Some Selected Drug Plants 554
Nouri Y. Mary, Bernard V. Christensen, and *Jack L. Beal*

Water-in-Oil Emulsifying Agents. VI. Esters Synthesized from Cholesterol Derivatives 558
E. L. Cataline, Joseph E. Sinsheimer, and *Lee Worrell*

Water-in-Oil Emulsifying Agents. VII. An Improved Method of Evaluating Emulsifying Efficiencies 562
Lee Worrell and *Joseph E. Sinsheimer*

The Effect of Age and of Heat on the "Ferment" in *Cascara sagrada* 569
M. Christine Gosselin and *Charles W. Bauer*

A Note on the Flavoring of Methionine and Choline Combinations 573
Herbert M. Gross

Book Notices 574

Advertising ..... Cover pages 2, 3, 4, and pages I to VIII

New and Nonofficial Remedies ..... IV and V

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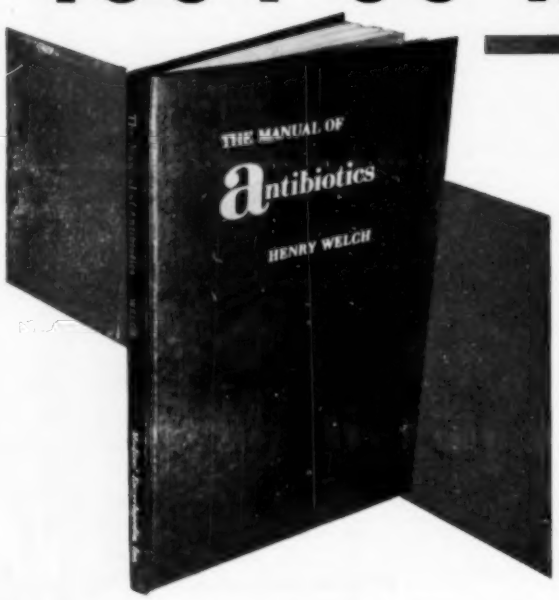

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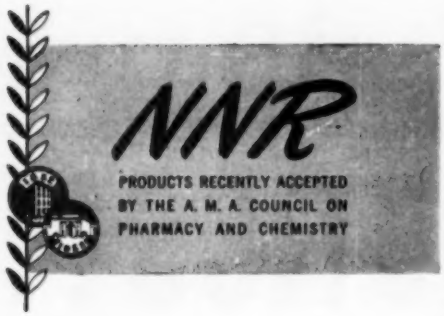
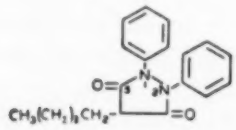


PHENYLBUTAZONE.—Butazolidin (Geigy.)—$C_{19}H_{20}N_2O_2$.—M. W. 308.37.—1,2-Diphenyl-4-butyl-3,5-pyrazolidinedione.—The structural formula of phenylbutazone may be represented as follows:



Actions and Uses.—Phenylbutazone, a synthetic pyrazolone derivative chemically related to aminopyrine, manifests similar analgesic and antipyretic properties in animals. In addition to this, it exerts an anti-inflammatory effect in delaying and minimizing local tissue reaction produced by chemical and physical irritants. Although its analgesic effect is less than that of acetylsalicylic acid in nonrheumatic conditions, phenylbutazone has been found to be clinically useful in the management of certain painful musculoskeletal disorders. Its mode of action in such conditions cannot be ascribed to a similarity with hormone drugs.

When administered orally, phenylbutazone is rapidly and completely absorbed; a single dose produces a peak plasma concentration in about two hours. When it is given intramuscularly as the sodium salt, the peak plasma level is usually not attained for six to ten hours. The delay might be the result of precipitation of the drug, which is insoluble at the normal *p*H of the tissues. Stable plasma levels from 65 to 140 mg. per liter usually are reached on the third or fourth day following daily doses of 0.6 to 0.8 Gm. After a single dose, approximately one-third is concentrated in the plasma and is bound, almost entirely, to plasma protein. Increased dosage (over 0.8 Gm. daily) is accompanied by a sharp increase in excretion of urinary metabolites, but with very little increase in the plasma concentration of the drug. This suggests that the protein-bound portion in the plasma acts as a drug depot and that, once the plasma proteins become saturated, the unbound excess is rapidly metabolized. At an oral dosage of 0.6 to 0.8 Gm. daily, the drug is metabolized at the rate of 15 to 25% per day, so that a period of seven to ten days usually elapses before the drug disappears from the blood stream. Phenylbutazone is not excreted as such in any significant amount, but at least three metabolites have been detected in the urine in significant quantity. The drug produces a temporary decrease in urine volume and significant retention of sodium and chlorine ions. Upon discontinuance there is a compensatory diuresis and liberation of the excess retained sodium chloride. Potassium excretion is unaffected. Endogenous creatinine clearance studies indicate that glomerular filtration also is not affected by phenylbutazone, suggesting that decreased excretion of water and salt results from tubular reabsorption.

Phenylbutazone is chiefly useful in the treatment of gout and, to a lesser extent, psoriasis with arthritis, ankylosing spondylitis, rheumatoid arthritis, and painful shoulder (peritendinitis, capsulitis, bursitis, and acute arthritis of that joint). In gout, the use of the drug is associated with a reduction in serum uric acid. As with other agents, replases are more prone to occur in rheumatoid conditions requiring continuous medication or alternative therapy. Phenylbutazone, because of the high incidence of untoward side effects, should not be used for the treatment of these conditions unless adequate trial of less hazardous therapeutic measures has proved unsuccessful. Its use in malum coxae senilis, osteoarthritis, osteoporosis, and mixed arthritis is not recommended because in these conditions the incidence of toxicity outweighs the degree of clinical improvement.

Phenylbutazone has untoward side effects in approximately 40% of patients, and it may be necessary to discontinue its use because of toxic effects in about 15%. The most frequently encountered reactions in the order of incidence include water retention (edema), nausea, rash, epigastric pain, vertigo, and stomatitis. Other less frequent but unusually severe reactions have been reported, including activation of peptic ulcer with bleeding, hepatitis, hypertension, transient psychosis, moderate leukopenia, agranulocytosis, thrombocytopenia, and purpura without thrombocytopenia. Other less commonly observed side effects include central nervous system stimulation, visual symptoms, anemia, lethargy, constipation, diarrhea, gastrointestinal hemorrhage, fever, and cardiac arrhythmia. Toxic side effects have been observed more frequently in women than in men.

Phenylbutazone is contraindicated in the presence of edema and in patients in whom there is danger of cardiac decompensation. Its use is inadvisable in patients with a history of peptic ulcer. Utmost caution is necessary when it is given to patients with a history of drug allergy or blood dyscrasia. In general, the use of phenylbutazone in conjunction with other potent drugs is not recommended because of the danger of increasing the incidence or severity of toxic reactions. The frequent occurrence of minor side effects and the occasional development of severe toxic manifestations require constant supervision of the patient by the physician. In addition to frequent clinical observations, weekly blood cell counts should be made during initial therapy and also at biweekly intervals when medication is continued over a prolonged period. Because sodium retention tends to occur, it is advisable to place patients on a restricted salt diet.

Dosage.—Phenylbutazone is administered orally. The recommended initial dosage is 0.3 to 0.8 Gm. daily, divided into three or four equal doses. Dos-

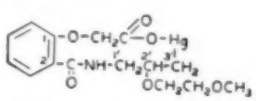
age in excess of 0.8 Gm. daily is inadvisable because this seldom produces greater therapeutic effect and may increase the toxic hazard. An average initial dosage of 0.6 Gm. daily, administered for one week, is considered adequate to determine the therapeutic effect of the drug. In the absence of a favorable response, further therapy should be discontinued. When improvement is obtained, the dosage should be gradually decreased to the minimal effective level. An effective maintenance dosage may be as low as 0.1 to 0.2 Gm. daily. In the treatment of transient conditions such as painful shoulder, medication should be discontinued a few days after relief of symptoms; in the event of relapse, therapy is resumed as needed to control symptoms. In the treatment of more chronic disorders, medication may be continuous at the minimal effective level required to maintain relief and freedom from acute exacerbations. Medication should be discontinued whenever toxic symptoms appear or whenever blood studies indicate any significant reduction in the formed elements. In some instances, therapy may be resumed at a lower dosage level when results of blood tests return to normal.

Tests and Standards.—

Physical Properties: Phenylbutazone is a white or very light yellow powder with a slightly bitter taste and a very slight aromatic odor, m. p. 103–106°. It is freely soluble in acetone, ether, and ethyl acetate and very slightly soluble in water. The amount that dissolves in alcohol to form 100 ml. of solution is 4.8 Gm. Phenylbutazone is stable if stored at room temperature in closed containers in the absence of moisture.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **15** , 1495(August 21, 1954).]

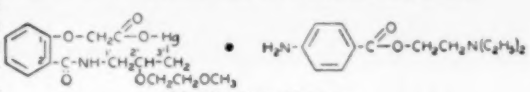
MERETHOXYLLINE.——$C_{15}H_{19}HgNO_5$.——M. W. 509.92.—Dehydro-2-[N-(3′-hydroxymercuri-2′-methoxyethoxy) propylcarbamyl] phenoxyacetic acid.—The structural formula of merethoxylline may be represented as follows:



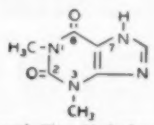
Physical Properties: Merethoxylline is a white, amorphous, almost odorless solid. It is freely soluble in alkalis, slightly soluble in acids, and very slightly soluble in ether. The approximate amount that dissolves at 25° in alcohol to form 100 ml. of solution is 0.15 Gm. Merethoxylline is stable to light and moisture at room temperature, but decomposes when heated to about 190°.

[For more information regarding this monograph see *J. Am. Med. Assoc.* **155,** 1497 (August 21, 1954).]

MERETHOXYLLINE PROCAINE.—Dicurin Procaine (Lilly).—$C_{15}H_{19}HgNO_5 . C_{13}H_{20}N_2O_2$.—M. W. 746.23.—Dehydro-2-[N-(3′-hydroxymercuri-2′-methoxyethoxy) propylcarbamyl] phenoxyacetic acid (merethoxylline), 2-diethylaminoethyl *p*-aminobenzoate (procaine), and theophylline.—Merethoxylline procaine consists of a mixture of 1 mole of the procaine salt of merethoxylline (the mercury compound, $C_{15}H_{19}HgNO_5$, M. W. 509.92) and 1.4 moles of anhydrous theophylline U. S. P. It is prepared by dissolving merethoxylline and procaine in water and then adding the theophylline. It is not isolated from solution. The structural formulas of the compounds may be represented as follows:



Merethoxylline Procaine



Theophylline (Anhydrous)

Actions and Uses.—Merethoxylline procaine has the same actions and uses as other mercurial diuretics. It is used to prevent or control excessive accumulation of fluid in the tissue spaces and body cavities as an adjunct in the management of congestive heart failure, cirrhosis of the liver with ascites, and nephrosis.

Merethoxylline procaine does not have greater toxicity than other organic mercurial compounds. The procaine component minimizes the discomfort of local irritation that may be produced by the mercurial compound when injected into the tissues, but such reactions are not encountered frequently. Like similar mercurial diuretics, it should not be administered in the presence of sensitivity to mercury or theophylline, acute glomerulonephritis, ulcerative colitis, or gouty diathesis. Sensitivity to procaine is also a contraindication, and, because of that component, physicans should be alert for possible signs of such a reaction in susceptible patients. As with any diuretic, it should be used with great caution in benign prostatic hypertrophy when distention of the bladder may precipitate obstruction or whenever the blood urea nitrogen exceeds 60 mg. per 100 cc. If albuminuria, hematuria, or oliguria appears after administration of the drug, it should be discontinued.

Dosage.—Merethoxylline procaine is administered intramuscularly or subcutaneously. Intravenous administration is not advised, because diuretic effectiveness is seldom greater and the risk of deleterious side effects is greatly increased. Subcutaneous injection should be deep because superficial deposit of the drug predisposes the patient to local reaction. The drug is injected as an aqueous solution containing 0.195 Gm. of the salt per cubic centimeter. This includes 0.1 Gm. of the organic mercurial component (equivalent to 39.3 mg. of mercury), 0.05 Gm. of theophylline, and 0.045 Gm. of procaine base. The initial dose should be 0.5 cc. of such solution to preclude a serious reaction resulting from idiosyncrasy. In adults, the average subsequent daily dosage is a single injection of 2 cc. preferably in the morning, or this can be administered as two injections of 1 cc. each. Alternative sites of injection should be used to reduce the possibility of local reactions.

Tests and Standards.—

For description and standards for theophylline U. S. P. see the *U. S. Pharmacopeia* under Theophylline. For tests and standards for merethoxylline N. N. R. and procaine N. N. R., see the monographs for these materials.

[For more information regarding this monograph see *J. Am. Med. Assoc.*, **155,** 1496 (August 21, 1954).]

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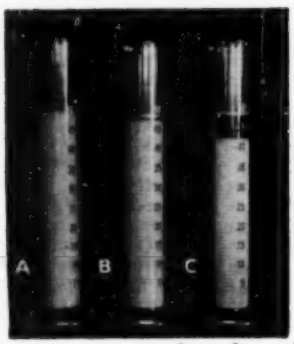

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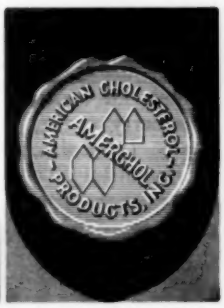

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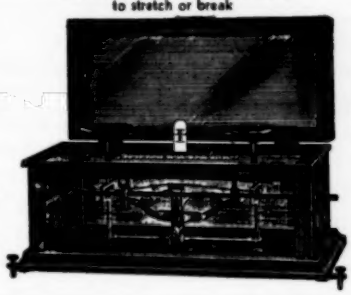

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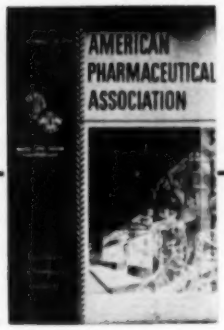

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▸ **Dactil,** states **Lakeside Laboratories,** is backed by proved performance, and usually relieves pain spasm in ten minutes.

▸ In two insertions, **Lederle Laboratories** delineates the broad-spectrum activity of **Achromycin** and **Achromycin Tablets.**

▸ **'Vi-Mix Drops'** are described as "an outstanding member of the **Lilly** family of vitamins."

▸ In one insertion **S. E. Massengill** directs attention to **Gallogen** for use in the treatment of diseases of the liver and in another, lists its prescription specialties: **Adrenosem, Aminodrox, Broniacin, Livitamin, Massengill Powder, Obedrin, Salcedrox,** and **Semhyten.**

▸ **Merck & Co.** stresses the value of its vitamin preparations in countering nutritional deficiencies.

▸ **Owens-Illinois** offers a complete selection of clear containers for dry prescriptions.

▸ **Abdol** is **Parke, Davis & Company's** new vitamin-mineral formula.

▸ **Pfizer Laboratories** observes that, "orally, parenterally or topically, there's a **Terramycin** dosage form for every need."

▸ Qualified students can matriculate at **Philadelphia College of Pharmacy and Science** for courses leading to B.Sc., M.Sc., and D.Sc. degrees. **P.C.P. & S.** also has schools of Chemistry, Bacteriology and Biology.

▸ **Robins** calls attention to the fact that **Pabalate** and **Pabalate-Sodium Free** "provide antirheumatic action closely interrelated with that of the cortisone-like steroids, yet without danger of adverse side effects."

▸ The **Roerig** announcement features **Tetracyn Intramuscular** and notes the expanding line of **Tetracyn** dosage forms.

▸ **Altepose** is described as "more effective than gymnastics" by **Sharpe & Dohme** for reducing diets.

▸ **Smith, Kline & French Laboratories** advises that **Benzedrex Inhaler** sales can be doubled with the new display carton.

▸ **Cheracol** is publicized by **Upjohn** as "the recognized leader . . . of expectorants and cough preparations."

▸ **Vi-Aquamin,** "the only aqueous vitamin-mineral product in a *single* capsule," is **U. S. Vitamin Corporation's** contribution.

▸ **Methium,** its new oral hypotensive agent, is the subject of the **Warner-Chilcott** insertion.

▸ For colds, sinusitis, and allergic rhinitis, **Winthrop-Stearns** suggests the following: **Neo-Synephrine HCl, Neo-Synephrine Nasal Spray, NTZ Nasal Solution,** and **NTZ Nasal Spray.**

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Isonicotinyl Hydrazones as Antitubercular Agents and Derivatives for Identification of Aldehydes and Ketones*

By PETER P. T. SAH and S. ANDERSON PEOPLES†

Isonicotinic acid hydrazide is found to be a good reagent for the identification of aldehydes and ketones. Of 106 carbonyl compounds tested, 93 form crystalline isonicotinyl hydrazones with distinct melting points; the remaining 13, which crystallize very slowly, may be converted into their hydrochlorides and identified. A large number of isonicotinyl hydrazones are found to possess very high *in vivo* activity against *Mycobacterium tuberculosis* H37Rv. They are apparently more effective than streptomycin and less toxic than isonicotinic acid hydrazide.

THE USE of *p*-nitrobenzhydrazide as a reagent for the identification of aldehydes and ketones has been investigated systematically (10, 21–23). *p* - Aminobenzhydrazide, *p*-acetaminobenzhydrazide, and their derivatives (24) have also been synthesized recently and their antitubercular activity studied. Since isonicotinic acid hydrazide bears a very close structural relationship to the above mentioned hydrazides, especially *p*-nitrobenzhydrazide (25), it seems interesting and important to prepare as many isonicotinyl hydrazones as possible in order to find out (*a*) if isonicotinic acid hydrazide as a reagent for the preparation of characteristic derivatives of aldehydes and ketones is comparable to *p*-nitrobenzhydrazide, and (*b*) whether some derivatives of isonicotinic acid hydrazide, as chemotherapeutic agents for the treatment of tuberculosis, are better than the uncombined parent drug.

Meyer and Mally (26) who first prepared isonicotinic acid hydrazide in 1912 described in their paper only a few derivatives, namely, the isonicotinyl hydrazones derived from benzaldehyde, *o*-chlorobenzaldehyde, and vanillin. Nothing was reported about their biological properties. Fox, *et al.* (27, 28), and Bernstein, *et al.*(29), who simultaneously and independently reported the antitubercular activity, *in vitro* as well as *in vivo*, of isonicotinic acid hydrazide, did not lay much emphasis on the isonicotinyl hydrazones, with the exception perhaps of the D-glucose derivative (30), which was tried and abandoned clinically because of its instability.

Shchukina, *et al.* (31), reported in 1952 the synthesis of a number of isonicotinyl hydrazones from aldehydes and ketones, namely, those derived from benzaldehyde, *p*-nitrobenzaldehyde, salicyl aldehyde, anisaldehyde, *p*-dimethylaminobenzaldehyde, vanillin, veratraldehyde, 2,-4-dihydroxybenzaldehyde, *p*-acetaminobenzaldehyde, acetophenone, cyclohexanone, and

* Received January 29, 1954, from the University of California School of Veterinary Medicine, Davis, Calif.

This is the 21st paper of the series on the use of hydrazides of organic acids as reagents for the identification of aldehydes and ketones. Previous publications on the subject matter are listed in the references (1–20).

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acetone. Their biological tests indicated that some of these isonicotinyl hydrazones are more active *in vivo* and far less toxic in animals (mice) infected with various strains of *Mycobacterium tuberculosis*. These authors lay great emphasis on the high toxicity of isonicotinic acid hydrazide, believing that the new drug does not possess a big margin of safety.

Yale, *et al.* (32), reported in 1953 the synthesis of a large number of isonicotinyl hydrazones, the antitubercular activities of which were simultaneously described by their colleagues (33). They drew the conclusion that none of these derivatives, as chemotherapeutic agents for tuberculosis, are better than the uncombined, free hydrazide of isonicotinic acid.

McMillan, *et al.* (34), prepared a number of isonicotinyl hydrazones (mostly derivatives of aliphatic aldehydes and ketones) and submitted them to biological tests. They reported that about 90 per cent of these derivatives had an antitubercular activity *in vivo* of the order of magnitude of that of the parent drug, isonicotinic acid hydrazide.

Sah recently synthesized D-glucuronolactone isonicotinyl hydrazone (35). The biological tests of this new drug on animals (36–38) and the clinical trials by several authoritative physicians in San Francisco (39) and in France (40) indicated that the new drug is very active and comparatively less toxic and therefore possibly a much superior chemotherapeutic agent for tuberculosis to streptomycin, *p*-acetaminobenzaldehyde thiosemicarbazone (Tibione), *p*-aminosalicylic acid, or isonicotinic acid hydrazide (Isoniazid).

An analog of the above derivative, namely, D-galacturonic acid isonicotinyl hydrazone, has also been prepared by Sah and Peoples and studied (41). The *in vivo* activity of this new drug against *Mycobacterium tuberculosis* H37Rv is about of the same order of magnitude as isonicotinic acid hydrazide. Its acute toxicity, however, is much lower than that of the free hydrazide. The LD_{50} of this new drug is 1.8 Gm. per Kg. mice given intravenously or intraperitoneally against that of 165 mg. of isonicotinic acid hydrazide per Kg. mice given intravenously or subcutaneously (42). Chronic toxicity studies in dogs by Peoples (38) indicate also that the new drug is only one-tenth as toxic as the parent drug, the free hydrazide.

Another very interesting derivative is found in the new compound, pyridoxal isonicotinyl hydrazone (43), which not only is active *in vivo* against *Mycobacterium tuberculosis* H37Rv, but also possesses the biological properties of vitamin B_6 (pyridoxine) (44). The new agent has also been found by Freedlander and Furst to show some significant acitivity against mammary cancer in mice and certain forms of leukemia in mice (45).

These recent results of biological tests indicate that if too much emphasis is laid on the value of isonicotinic acid hydrazide alone as a chemotherapeutic agent for the treatment of tuberculosis and overlooking the possibilities of its derivatives, there is the great danger that some most interesting and valuable compounds might be missed.

This paper aims to report (*a*) the use of isonicotinic acid hydrazide as a reagent for the preparation of characteristic derivatives in the identification of aldehydes and ketones and (*b*) the very high antitubercular activity *in vivo* of a large number of isonicotinyl hydrazones that we synthesized for the purpose of characterization.

Acute and chronic toxicity studies of these active isonicotinyl hydrazones are being carried out in our laboratory. Results will later be published.

EXPERIMENTAL

General Procedure for the Preparation of Isonicotinyl Hydrazones of Aldehydes and Ketones for the Purpose of Identification.—The reagent, isonicotinic acid hydrazide (0.7 Gm. or 0.005 mole), in a small Erlenmeyer flask was dissolved in distilled water (10 cc.) at 60°, by slight warming on the steam bath. The aldehyde or ketone, in slight excess over the molecular equivalent (0.006 to 0.01 mole) in absolute ethanol (10 cc.) solution, was added. The mixture was gently boiled on the steam bath for five minutes and then allowed to stand quietly for twenty-four to seventy-two hours at room temperature.

The crystalline isonicotinyl hydrazones that separated were then filtered off with suction, sucked practically to dryness, and then recrystallized from a suitable solvent (usually methanol, a mixture of methanol and benzene, or a mixture of methanol and petroleum ether). Table I shows the yields, melting points, and appearance of crystals of these derivatives. Table II summarizes the chemical analyses for carbon, hydrogen, and nitrogen present in these derivatives, thus proving the purity of these preparations.

Acetone Isonicotinyl Hydrazone.—This derivative was prepared more conveniently by the following procedure, applicable to carbonyl compounds with boiling points lower than or near to 100°, such as acetaldehyde, isobutyraldehyde, isovaleraldehyde, methyl ethyl ketone, and diethyl ketone. In a small Erlenmeyer flask, isonicotinic acid hydrazide (1.4 Gm.) was covered with acetone (15 cc.). The mixture was shaken for five minutes and then allowed to stand overnight. The reaction mixture was then poured into a small evaporating dish and heated on the steam bath till all the solvent was evaporated off. The crude product (100% yield), m. p. 159–160°, was recrystallized from a minimum

TABLE I.—YIELDS* AND PHYSICAL PROPERTIES* OF ISONICOTINYL HYDRAZONES DERIVED FROM ALDEHYDES AND KETONES

	Isonicotinyl Hydrazones Derived from	Yield, %	Appearance of Crystals	M. p. (Uncorr.)	° C. (Corr.)	Number of Recrystallizations Required
1	Benzaldehyde (31, 32)	80	White needles and plates;[a] rods[b]	197–198	201–202	Twice
2	2-Methylbenzaldehyde	90	White platelets[c]	165–167	168–170	Twice to thrice
3	3-Methylbenzaldehyde	90	White prisms[c]	178–180	181–182	Twice to thrice
4	4-Methylbenzaldehyde	90	White rods[c]	163–165	165–166	Twice
5	Cuminaldehyde	75	White, thick plates[a, d]	135–136	137–138	Thrice
6	2-Nitrobenzaldehyde	99	Yellow plates;[a] yellow prisms[b]	229–230	235–236	Twice
7	3-Nitrobenzaldehyde	98	Yellow plates;[a] white rods[b]	226–227	231–232	Twice
8	4-Nitrobenzaldehyde (31)	90	Yellow, short rods[a, b]	275–276	281–282	Once to twice
9	2-Chlorobenzaldehyde (26)	99	White platelets;[a] white prisms[b]	216–217	221–222	Twice
10	4-Chlorobenzaldehyde	100	White, large, thick plates;[a] needles[b]	212–213	217–218	Once to twice
11	2,4-Dichlorobenzaldehyde	100	White, fine needles[a, b]	222–223	228–229	Once to twice
12	3,4-Dichlorobenzaldehyde	100	White plates;[a] white rhombs[b]	241–242	247–248	Once to twice
13	2-Hydroxybenzaldehyde (31, 55)	100	White rods;[a, b] needles[e]	245–246	252–253	Twice
14	3-Hydroxybenzaldehyde	95	Pale yellow platelets;[a] white rods[b]	260–261	268–269	Twice
15	4-Hydroxybenzaldehyde (31, 55)	99	Pale yellow rods;[a] white prisms[b]	285–286	293–294	Twice
16	2-Methoxybenzaldehyde	99	Pale yellow rods;[a] white prisms[b]	187–188	190–191	Once to twice
17	4-Methoxybenzaldehyde (31)	99	White needles;[a] white rods[b]	169–170	172–173	Twice to thrice
18	4-Dimethylaminobenzaldehyde (31)	99	Golden yellow rods;[a] yellow, thick plates[b]	201–202	206–207	Twice
19	4-Diethylaminobenzaldehyde (32)	99	Yellow rods;[a] yellow prisms[b]	185–186	188–189	Twice
20	Cinnamaldehyde	99	Yellow, thick plates[a, b]	197–198	201–202	Twice
21	Hydrocinnamaldehyde	63	White, narrow plates;[a] white needles[b]	103–104	104–105	Thrice
22	2-Hydroxy-1-naphthaldehyde	100	Greenish yellow rods;[a] greenish yellow needles[b]	260–261	268–269	Twice
23	Vanillin (26, 31)	99	Pale yellow plates;[a] pale yellow rods[b]	226–227	232–233	Once to twice
24	Veratraldehyde (31)	72	White, large prisms[a, b]	187–188	190–191	Twice
25	Piperonal	99	White platelets;[a] white rods[b]	231–232	237–238	Twice
26	2-Ethoxybenzaldehyde	90	White, thick plates;[a] prisms[b]	199–200	204–205	Twice to thrice
27	2-Hydroxy-5-chlorobenzaldehyde	95	White prismatic needles;[a] white rods and prisms[b]	241–242	248–249	Once to twice
28	2-Hydroxy-5-bromobenzaldehyde	100	White needles;[a] white, narrow plates[b]	254–255	262–263	Once to twice
29	2-Hydroxy-3-methoxybenzaldehyde	99	White rods or narrow plates;[a] white prismatic needles[b]	230–231	236–237	Twice
30	2,4-Dihydroxybenzaldehyde	99	Yellow, thick plates[a, b]	284–295 dec.	294–295 dec.	Twice
31	3-Ethoxy-4-hydroxybenzaldehyde	99	Pale yellow platelets;[a] pale yellow rods[b]	251–252	258–259	Once to twice
32	3,4-Diethoxybenzaldehyde	94	White needles;[a] thick plates and prisms[b]	189–190	193–194	Once to twice

TABLE I (*continued*)

	Isonicotinyl Hydrazones Derived from	Yield, %	Appearance of Crystals	M. P. (Uncorr.)	° C. (Corr.)	Number of Recrystallizations Required
33	α-n-Amylcinnamaldehyde	65	White prisms[a, c]	85–86	85–86	Twice to thrice
34	Furfural	70	White, thin plates;[a] long, narrow plates[b]	210–211	215–216	Twice
35	5-Nitrofurfural	80	Brownish yellow leaflets[a, b]	249–250	256–257	Twice
36	Furylacrolein	89	Yellow rods[a, b]	233–234	239–240	Once to twice
37	n-Heptaldehyde	Poor	White, small needles[a, f]	97–98	98–99	Thrice
38	Acetaldehyde (32)	70	White needles[a, d]	173–174	176–177	Twice to thrice
39	4-Acetaminobenzaldehyde (31, 32)	100	White, small prisms[a, b]	283–284	292–293	Once to twice
40	2-Formylthiophene	89	Brown prisms;[a] yellow prisms[b]	234–235	240–242	Once to twice
41	D-Glucuronolactone (35)	75	White plates and rods[b, e]	150–160 foaming and decomposition		Twice to thrice
42	D-Galacturonic acid (41)	92	White, thin plates;[a] white platelets[g]	207–208	212–213	Once to twice
43	4-Bromobenzaldehyde	100	White, thick plates or prisms[a, b]	216–217	222–223	Once
44	4-Benzenesulfonylbenzaldehyde	50	White needles;[a] white prisms[b]	214–215	219–220	Thrice
45	Acetophenone (31, 34)	99	White rods[a, b]	172–173	175–176	Once to twice
46	4-Methylacetophenone	99	White needles[a, b]	189–190	193–194	Twice
47	4-Nitroacetophenone	99	Practically white needles[a, b, e]	274–275	281–282	Twice to thrice
48	3-Nitroacetophenone	99	White, fine needles[a, b]	218–219	223–224	Twice
49	4-Chloroacetophenone	99	White needles[a, b]	239–240	245–246	Twice
50	4-Bromoacetophenone	100	White needles[a, b]	250–251	257–258	Once to twice
51	4-Methoxyacetophenone	89	White rods;[a] white needles[b]	193–194	197–198	Twice to thrice
52	4-Aminoacetophenone	67	Yellow plates;[a] yellow needles[b]	227–228	231–232	Once to twice
53	3-Aminoacetophenone	60	White, large, thick plates;[a] white prismatic needles[b]	196–197	201–202	Once to twice
54	2-Hydroxyacetophenone	73	White, thick platelets or rods[a, b]	217–218	223–224	Once to twice
55	4-Hydroxyacetophenone	94	White plates[a, e]	269–270	280–281	Twice or thrice
56	3-Hydroxyacetophenone	99	Yellow, thick plates or prisms;[a] yellow plates[b]	228–229	231–232	Once to twice
57	4-Ethoxyacetophenone	99	White rods;[a] white needles[b]	201–202	205–206	Twice
58	4-Ethylacetophenone	99	White rods and narrow plates[a, b]	174–175	177–178	Twice
59	2,5-Dimethylacetophenone	75	White, small short needles;[a] white thin plates[f]	113–114	114–115	Thrice
60	2-Methyl-5-isopropylacetophenone	75	White, small needles;[a] white, thin plates[f]	125–126	127–128	Thrice
61	2,4-Dihydroxyacetophenone	80	White platelets or needles[a, b]	282–283	292–293	Thrice
62	4-Phenylacetophenone	100	White plates;[a] white platelets[b]	255–256	262–263	Twice
63	Propiophenone	95	White plates;[a] white prisms[d]	189–190	194–195	Twice
64	Phenyl n-propyl ketone	41	White rods;[a] white, small needles[d]	139–140	141–142	Thrice
65	Phenyl n-butyl ketone	40	White, narrow plates;[a] white rods[d]	123–124	125–126	Thrice
66	4-Hydroxypropiophenone	66	White rods;[a] white needles[b]	235–236	242–243	Twice to thrice
67	4-Methoxypropiophenone	96	White plates;[a] white prisms[c]	180–181	183–184	Twice
68	4-Chloropropiophenone	99	White, narrow plates;[a] white rods[b]	155–156	158–159	Twice

TABLE I (*continued*)

	Isonicotinyl Hydrazones Derived from	Yield, %	Appearance of Crystals	M. p. (Uncorr.)	° C. (Corr.)	Number of Recrystallizations Required
69	4-Aminopropiophenone	67	Pale yellow plates;[a] yellow prisms[b]	139–140	141–142	Twice
70	3-Methoxy-4-hydroxyacetophenone	65	White, short rods;[a] white needles[b]	213–214	219–220	Twice
71	1-Acetylnaphthalene	45	White, needles;[a] white, narrow plates[b]	215–216	220–221	Twice
72	2-Acetyl-1-naphthol	50	Greenish yellow needles or rods[a, b]	95–96	96–97	Twice
73	Acetone (31, 32, 34)	80	White prismatic needles from acetone	161–162	164–165	Once
74	Methyl ethyl ketone (32, 34)	99	White needles[d]	95–96	96–97	Once
75	Benzalacetone	99	Yellow rods[a, b]	186–187	190–191	Once
76	Anisalacetone (4-methoxybenzalacetone)	99	Yellow, thick plates;[a] yellow needles[b]	193–194	197–198	Once to twice
77	Vanillalacetone	99	Yellow, fine needles;[a] yellow rods[b]	223–224	228–229	Twice
78	Furfuralacetone	99	Yellow rods[a, e]	182–183	186–187	Once
79	Salicylideneacetone	99	Red prisms;[a] red platelets[b]	177–178	181–182	Twice
80	Phenoxyacetone	75	White plates[a, b]	134–135	136–137	Twice
81	Desoxybenzoin	99	White needles[a, b]	152–153	154–155	Twice
82	Benzoin	50	White prismatic needles[a, b]	134–135	136–137	Once
83	Cyclopentanone	Poor	Yellow prisms[a, b]	118–119	120–121	Once
84	Cyclohexanone (31, 32)	76	White prisms;[a] white rods[b, c]	165–166	168–169	Thrice
85	Isophorone	80	Yellow needles;[a] white needles[b]	180–181	184–185	Twice
86	β-Ionone	68	White needles,[a, b] turning yellow on standing	175–176	178–179	Twice
87	Ethyl benzoylacetate	81	Yellow rods;[a] yellow plates[c]	131–132	133–134	Twice
88	Levulinic acid	99	White, thick plates;[a] white prisms[g]	213–214	218–219	Once
89	Ethyl levulinate	84	White plates[a, e]	74–75	74–75	Twice
90	Diethyl ketone (32)	85	White needles[a, d, f]	86–87	86–87	Twice to thrice
91	Pinacolone (34)	90	White needles[a, d, f]	116–117	118–119	Twice
92	Isobutyraldehyde (32, 34)	82	White needles[a, d, f]	136–137	139–140	Twice to thrice
93	Isovaleraldehyde (34)	70	White needles[a, d, f]	115–116	117–118	Thrice

* Preliminary results were obtained by C. Y. Chiao, technician, and Dr. C. Wang, Associate Professor of Organic Analysis, The Catholic University, Peiping, China, 1940–1945. The results were thoroughly checked by one of the authors (Peter P. T. Sah) at U. C. College of Pharmacy, San Francisco, Calif., 1951–1953, and at U. C. School of Veterinary Medicine, Davis, California, 1953–1954.

[a] Recrystallized from 50% EtOH.
[b] MeOH.
[c] Mixture of MeOH and Benzene.
[d] Benzene.
[e] EtOH.
[f] Petroleum Ether.
[g] H_2O.

amount of boiling acetone. It formed beautiful white needles from acetone, m. p. 161–162° (uncorr.); 164–165° (corr.). Yield of the purified product: 1.45 Gm. (80% of the theoretical).

Levulinic Acid Isonicotinyl Hydrazone.—This interesting derivative was best and most easily prepared in water solution by the following simple procedure: Isonicotinic acid hydrazide (0.7 Gm.) was dissolved in distilled water (10 cc.). To the hot solution, levulinic acid (0.6 Gm.) was added. The solution was boiled for five minutes on the hot plate and then allowed to stand for twenty-four hours in the refrigerator. Beautiful, white crystals in the form of thick plates and prisms separated out quantitatively. They were then filtered off with suction, washed with ice-cold distilled water, and sucked to dryness. The dried product weighed 1.18 Gm. and showed a melting point of 211–212° (uncorr.): 216–217° (corr.). For purification, the crude product was dissolved in 50 cc. of boiling distilled water and filtered. On standing and cooling white prisms separated. The crystals were filtered off with suction, sucked to dryness, and finally dried in a vacuum desiccator over anhydrous calcium chloride for three days. The purified product weighed more than 1.15 Gm. and showed a melting point of 213–214° (uncorr.); 218–219° (corr.). Despite its low solubility in cold water, this new derivative is soluble in boiling water and very soluble in dilute acids or dilute alkalies (5% NaOH or 5% $NaHCO_3$). The sodium bicarbonate solution of the new drug is suitable for the purpose of intravenous injection. The above procedure can be used also for the preparation of the most active drug, namely,

D-galacturonic acid isonicotinyl hydrazone (38, 41), as well as other isonicotinyl hydrazones derived from water-soluble aldehydic or ketonic acids.

Hydrochlorides of Isonicotinyl Hydrazones of Some Aldehydes and Ketones.—The following aldehydes and ketones were found to form isonicotinyl hydrazones which were either too slow to crystallize or did not crystallize at all: phenylacetaldehyde, 2,3-dimethoxybenzaldehyde, citral, citronellal, phenylacetone, dibenzyl ketone, 4-methyl cyclohexanone, 3-methyl cyclohexanone, 2-methyl cyclohexanone, DL-menthone, *alpha*-ionone, methyl-*n*-hexyl ketone, ethyl acetoacetate, and ethyl levulinate. The products, however, were easily converted into their hydrochlorides which precipitated out very readily from the absolute ethyl alcohol solution in the crystalline forms. The following is the general procedure for the preparation of the hydrochlorides of isonicotinyl hydrazones:

Isonicotinic acid hydrazide (0.005 mole) was dissolved in absolute ethyl alcohol (25 cc.) by warming on the steam bath. To the solution, the aldehyde or ketone mentioned above, in slight excess over the molecular equivalent (0.0075 mole) dissolved in absolute ethyl alcohol (15 cc.) was added. The mixture was boiled on the steam bath for five minutes. After standing and cooling, a mixture of concentrated hydrochloric acid (2 cc.) and absolute ethyl alcohol (8 cc.) was added. The contents in the flask were agitated thoroughly. On standing for twenty-four hours in the refrigerator, the hydrochloride crystallized out. The white crystals were

TABLE II.—CHEMICAL ANALYSES OF ISONICOTINYL HYDRAZONES[a]

INH No.	Formula	% C		% H		% N	
		Calcd.	Found	Calcd.	Found	Calcd.	Found
1	$C_{13}H_{11}ON_3$	69.31	69.25	4.93	5.01	18.66	18.82
			69.19		4.88		18.75
2	$C_{14}H_{13}ON_3$	70.26	70.33	5.49	5.38	17.56	17.78
			70.41		5.25		17.74
3	$C_{14}H_{13}ON_3$	70.26	70.42	5.49	5.66	17.56	17.44
			70.19		5.52		17.52
4	$C_{14}H_{13}ON_3$	70.26	70.30	5.49	5.37	17.56	17.63
			70.42		5.50		17.71
5	$C_{16}H_{17}ON_3$	71.87	71.74	6.42	6.39	15.72	15.82
			71.69		6.51		15.90
6	$C_{13}H_{10}O_2N_4$	57.77	57.65	3.74	3.80	20.73	20.85
			57.62		3.67		20.91
7	$C_{13}H_{10}O_2N_4$	57.77	57.59	3.74	3.66	20.73	20.77
			57.70		3.78		20.82
8	$C_{13}H_{10}O_2N_4$	57.77	57.75	3.74	3.75	20.73	20.69
			57.72		3.79		20.68
9	$C_{13}H_{10}ON_3Cl$	60.11	59.96	3.89	3.95	16.18	16.20
			60.06		4.01		16.31
10	$C_{13}H_{10}ON_3Cl$	60.11	59.89	3.89	3.92	16.18	16.16
			59.92		3.86		16.21
11	$C_{13}H_9ON_3Cl_2$	53.07	53.11	3.09	2.97	14.29	14.33
			53.09		3.13		14.42
12	$C_{13}H_9ON_3Cl_2$	53.07	53.02	3.09	2.95	14.29	14.22
			52.98		3.17		14.15
13	$C_{13}H_{11}O_2N_3$	64.71	64.54	4.60	4.72	17.42	17.39
			64.63		4.83		17.53
14	$C_{13}H_{11}O_2N_3$	64.71	64.53	4.60	4.72	17.42	17.50
			64.61		4.83		17.38
15	$C_{13}H_{11}O_2N_3$	64.71	64.78	4.60	4.57	17.42	17.63
			64.82		4.74		17.59
16	$C_{14}H_{13}O_2N_3$	65.86	65.69	5.14	5.22	16.46	16.44
			65.74		5.30		16.45
17	$C_{14}H_{13}O_2N_3$	65.86	65.73	5.14	4.98	16.46	16.62
			65.91		5.06		16.53
18	$C_{13}H_{16}ON_4$	67.13	67.02	6.02	5.96	20.88	21.01
			66.99		6.12		20.95
19	$C_{17}H_{20}ON_4$	68.88	68.75	6.81	6.77	18.91	19.03
			68.91		6.68		19.00
20	$C_{19}H_{15}ON_3$	71.68	71.71	5.22	5.40	16.72	16.83
			71.59		5.37		16.78
21	$C_{18}H_{15}ON_3$	71.11	70.95	5.98	6.02	16.59	16.63
			70.99		6.08		16.71
22	$C_{17}H_{13}O_2N_3$	70.08	69.88	4.51	4.56	14.43	14.53
			69.97		4.63		14.48
23	$C_{14}H_{13}O_2N_3$	61.98	62.03	4.84	4.77	15.49	15.64
			61.89		4.93		15.58
24	$C_{15}H_{15}O_2N_3$	63.14	63.08	5.31	5.50	14.73	14.82
			62.97		5.43		14.78
25	$C_{14}H_{11}O_2N_3$	62.44	62.33	4.13	4.25	15.61	15.74
			62.28		4.22		15.71
26	$C_{15}H_{13}O_2N_3$	66.88	66.72	5.63	5.74	15.61	15.75
			66.91		5.68		15.82
27	$C_{13}H_{10}O_2N_3Cl$	56.63	56.60	3.66	3.72	15.24	15.33
			56.58		3.75		15.27

TABLE II (*continued*)

INH No.	Formula	% C		% H		% N	
		Calcd.	Found	Calcd.	Found	Calcd.	Found
28	$C_{12}H_{10}O_2N_2Br$	48.76	48.67	3.15	3.22	13.13	13.08
			48.59		3.31		13.23
29	$C_{14}H_{13}O_2N_3$	61.98	61.79	4.84	4.75	15.49	15.52
			61.88		4.81		15.44
30	$C_{13}H_{11}O_3N_3$	60.69	60.53	4.32	4.44	16.34	16.45
			60.57		4.52		16.50
31	$C_{15}H_{15}O_2N_3$	63.14	63.05	5.31	5.52	14.73	14.83
			62.98		5.48		14.69
32	$C_{17}H_{19}O_2N_3$	65.15	65.03	6.12	6.35	13.41	13.50
			64.97		6.28		13.46
33	$C_{20}H_{23}ON_3$	74.72	74.58	7.23	7.35	13.07	12.96
			74.55		7.41		13.02
34	$C_{11}H_9O_2N_3$	61.38	61.27	4.22	4.18	19.53	19.53
			61.33		4.25		19.56
35	$C_{11}H_8O_4N_4$	50.77	50.64	3.10	3.32	21.53	21.59
			50.59		3.27		21.65
36	$C_{12}H_{11}O_2N_3$	64.71	64.65	4.61	4.73	17.42	17.61
			64.59		4.77		17.56
37	$C_{13}H_{19}ON_3$	66.91	66.77	8.22	8.35	18.01	17.89
			66.83		8.41		17.92
38	$C_8H_9ON_3$	58.88	59.08	5.56	5.62	25.75	25.63
			58.99		5.58		25.57
39	$C_{15}H_{14}O_2N_4$	63.81	63.63	5.01	5.20	19.85	19.93
			63.74		5.15		20.02
40	$C_{11}H_9ON_3S$	57.12	57.05	3.93	4.04	18.17	18.22
			57.14		4.01		18.25
41	$C_{12}H_{12}O_6N_2$	48.79	48.58	4.45	4.33	14.24	14.51
			48.63		4.29		14.48
42	$C_{12}H_{15}O_7N_3$	46.00	45.83	4.83	5.00	13.42	13.52
			45.91		4.92		13.56
43	$C_{13}H_{10}ON_3Br$	51.33	51.11	3.31	3.10	13.82	13.95
			51.20		3.24		14.02
44	$C_{19}H_{15}O_2N_3S$	62.45	62.33	4.14	3.98	11.50	11.72
			62.26		4.07		11.65
45	$C_{14}H_{13}ON_3$	70.26	70.13	5.49	5.53	17.56	17.64
			69.97		5.62		17.73
46	$C_{15}H_{15}ON_3$	71.11	71.04	5.98	6.06	16.59	16.65
			70.96		6.11		16.72
47	$C_{14}H_{12}O_3N_4$	59.14	58.94	4.26	4.44	19.71	19.83
			59.02		4.38		19.91
48	$C_{14}H_{12}O_3N_4$	59.14	59.21	4.26	4.35	19.71	19.66
			59.08		4.29		19.62
49	$C_{14}H_{12}ON_3Cl$	61.48	61.33	4.43	4.61	15.35	15.44
			61.28		4.57		15.39
50	$C_{14}H_{12}ON_3Br$	52.84	52.73	3.81	3.92	13.20	13.34
			52.69		3.85		13.28
51	$C_{15}H_{15}O_2N_3$	66.89	66.75	5.62	5.73	15.60	15.58
			66.66		5.81		15.52
52	$C_{14}H_{14}ON_4$	66.11	66.03	5.56	5.72	22.03	21.95
			66.08		5.69		21.88
53	$C_{14}H_{14}ON_4$	66.11	66.06	5.56	5.65	22.03	22.09
			65.97		5.68		21.93
54	$C_{14}H_{13}O_2N_3$	65.86	65.75	5.14	5.23	16.46	16.52
			65.72		5.31		16.47
55	$C_{14}H_{13}O_2N_3$	65.86	65.77	5.14	5.00	16.46	16.54
			65.91		4.98		16.63
56	$C_{14}H_{13}O_2N_3$	65.86	65.65	5.14	5.08	16.46	16.56
			65.71		5.02		16.61
57	$C_{16}H_{17}O_2N_3$	67.81	67.68	6.06	5.92	14.83	14.74
			67.95		6.11		14.69
58	$C_{16}H_{17}ON_3$	71.87	71.78	6.42	6.33	15.72	15.84
			71.82		6.29		15.78
59	$C_{16}H_{17}ON_3$	71.87	71.66	6.42	6.27	15.72	15.83
			71.73		6.32		15.90
60	$C_{18}H_{21}ON_3$	73.19	72.89	7.15	7.02	14.23	14.38
			72.94		6.98		14.42
61	$C_{14}H_{12}O_2N_2$	61.97	62.03	4.84	4.92	15.49	15.55
			61.89		5.01		15.63
62	$C_{20}H_{17}ON_3$	76.16	76.08	5.44	5.63	13.32	13.44
			75.97		5.59		13.38
63	$C_{15}H_{15}ON_3$	71.11	71.08	5.98	6.03	16.59	16.72
			71.22		6.11		16.67
64	$C_{16}H_{17}ON_3$	71.87	71.79	6.42	6.53	15.72	15.68
			71.82		6.60		15.85

TABLE II (*continued*)

INH No.	Formula	% C Calcd.	% C Found	% H Calcd.	% H Found	% N Calcd.	% N Found
65	$C_{17}H_{13}ON_3$	72.56	72.44	6.82	6.91	14.94	14.88
			72.39		6.94		14.83
66	$C_{16}H_{15}O_2N_3$	66.89	66.91	5.62	5.72	15.60	15.53
			66.79		5.68		15.47
67	$C_{16}H_{17}O_2N_3$	67.81	67.72	6.06	6.02	14.83	14.75
			67.67		5.96		14.69
68	$C_{15}H_{14}ON_3Cl$	62.60	62.48	4.91	5.00	14.60	14.72
			62.51		5.03		14.67
69	$C_{15}H_{16}ON_4$	67.13	67.04	6.02	5.98	20.88	20.95
			66.96		6.11		21.04
70	$C_{15}H_{15}O_3N_3$	63.14	62.93	5.31	5.45	14.73	14.75
			63.05		5.55		14.81
71	$C_{18}H_{15}ON_3$	74.71	74.66	5.24	5.13	14.52	14.63
			74.59		5.08		14.57
72	$C_{18}H_{15}O_2N_3$	70.80	70.73	4.96	4.82	13.76	13.83
			70.68		4.77		13.79
73	$C_9H_{11}ON_3$	60.99	61.11	6.27	6.18	23.71	23.66
			61.02		6.23		23.74
74	$C_{10}H_{13}ON_3$	62.78	62.83	6.86	6.75	21.97	21.89
			62.91		6.69		22.02
75	$C_{16}H_{15}ON_3$	72.42	72.37	5.71	5.63	15.84	15.91
			72.29		5.58		16.00
76	$C_{17}H_{17}O_2N_3$	69.12	68.89	5.81	5.78	14.23	14.32
			68.95		5.85		14.27
77	$C_{17}H_{15}O_2N_3$	65.57	65.65	5.51	5.38	13.50	13.47
			65.49		5.43		13.61
78	$C_{14}H_{13}O_2N_3$	65.89	66.03	5.13	5.22	16.46	16.55
			65.99		5.08		16.62
79	$C_{16}H_{15}O_2N_3$	68.30	68.22	5.38	5.44	14.94	15.08
			68.17		5.53		15.17
80	$C_{15}H_{13}O_2N_3$	66.89	66.96	5.62	5.55	15.61	15.59
			66.77		5.48		15.70
81	$C_{20}H_{17}ON_3$	76.16	75.98	5.44	5.33	13.33	13.42
			76.04		5.37		13.51
82	$C_{20}H_{17}O_2N_3$	72.48	72.39	5.18	5.04	12.68	12.72
			72.41		5.11		12.80
83	$C_{11}H_{12}ON_2$	64.99	65.12	6.46	6.29	20.68	20.73
			65.06		6.33		20.69
84	$C_{12}H_{13}ON_3$	66.32	66.41	6.97	6.89	19.34	19.45
			66.37		7.02		19.53
85	$C_{13}H_{19}ON_3$	70.00	69.89	7.46	7.39	16.33	16.48
			69.92		7.36		16.53
86	$C_{13}H_{21}ON_3$	73.26	73.31	8.11	8.05	13.49	13.44
			73.22		8.13		13.38
87	$C_{17}H_{17}O_3N_3$	65.57	65.62	5.51	5.35	13.50	13.37
			65.60		5.42		13.41
88	$C_{11}H_{12}O_3N_2$	56.15	56.06	5.58	5.43	17.86	17.68
			56.12		5.39		17.72
89	$C_{12}H_{17}O_2N_3$	59.29	59.18	6.52	6.39	15.96	16.03
			59.25		6.44		16.11
90	$C_{11}H_{15}ON_3$	64.37	64.25	7.38	7.25	20.47	20.53
			64.44		7.19		20.61
91	$C_{12}H_{17}ON_3$	65.71	65.65	7.83	7.70	19.16	19.25
			65.59		7.75		19.31
92	$C_{10}H_{13}ON_3$	62.78	62.75	6.86	6.95	21.97	21.78
			62.82		6.66		21.85
93	$C_{11}H_{15}ON_3$	64.37	64.53	7.38	7.65	20.47	20.61
			64.46		7.62		20.58

[a] For INH number, refer to Table I.

Preliminary results were obtained by C. Y. Chiao, technician, and Dr. C. Wang, Associate Professor of Organic Analysis, The Catholic University, Peiping, China, 1940–1945. The results were thoroughly checked by one of the authors (Peter P. T. Sah) at U. C. College of Pharmacy, San Francisco, Calif., 1951–1953, and at U. C. School of Veterinary Medicine, Davis, California, 1953–1954.

filtered off with suction and then recrystallized from a minimum amount of boiling absolute ethyl alcohol. The physical properties and chemical analyses are summarized in Table III.

The hydrochlorides of isonicotinyl hydrazones, in general, are soluble in distilled water, slightly soluble in boiling absolute ethyl alcohol, but insoluble in the cold. Absolute ether, benzene, or petroleum ether are also solvents that will not dissolve the hydrochlorides.

Antitubercular Activity in vivo of Isonicotinyl Hydrazones in Mice Infected with Mycobacterium Tuberculosis H37Rv.—The biological tests were performed according to the standard methods described in the current literature (46–54), with slight modifications in order to bring out a sharper con-

TABLE III.—YIELDS, PHYSICAL PROPERTIES, AND CHEMICAL ANALYSES OF HYDROCHLORIDES OF ISONICOTINYL HYDRAZONES

Hydrochlorides of Isonicotinyl Hydrazones from	Yield %	Dec. Points, °C. (Corr.)	Appearance of Crystals	Cl, % Calcd.	Cl, % Found
Phenylacetaldehyde	70	245–246	Pale yellow needles[a]	12.86	13.01 12.95
2,3-Dimethoxybenzaldehyde	75	229–230	Almost white, small prisms[a]	11.02	11.22 11.18
Citral	65	271–275 (charred)	Pale yellow rods[b]	11.52	11.73 11.75
Citronellal	72	289–290	White needles[b]	11.45	11.65 11.71
Phenylacetone	75	279–280	White needles[a]	12.24	12.39 12.41
Dibenzyl ketone	76	275–276	White, featherlike needles[a]	9.69	9.93 9.85
4-Methylcyclohexanone	78	268–270	White, small needles[a]	13.24	13.45 13.41
3-Methylcyclohexanone	64	285–290 (charred)	White platelets[a]	13.24	13.55 13.49
2-Methylcyclohexanone	55	285–286 (charred)	White rods[a]	13.24	13.47 13.49
DL-Menthone	62	296–300 (charred)	White rods[b]	11.45	11.72 11.68
α-Ionone	68	245–246 (foaming)	Yellow platelets[b]	10.19	10.42 10.37
Methyl-*n*-hexyl ketone	65	306–310 (charred)	White, small prisms[b]	12.49	12.75 12.67
Ethyl acetoacetate	55	Decomposition started at 220 and completed between 275–285	White platelets[a]	12.41	12.63 12.55
Ethyl levulinate	65	266–268	White, small needles[a]	11.83	11.98 12.00

[a] Recrystallized from Ab. EtOH.
[b] 95% EtOH.

trast between the isonicotinyl hydrazones and the standard drugs for comparison, namely, streptomycin and isonicotinic acid hydrazide. The period of experimentation was set to last between four and five weeks instead of between two and three weeks. The experimental animals, no matter whether they had died during the testing period or were still living at the end of the experiment, were all submitted to careful autopsy and their internal organs examined for tuberculous infections. In this way, it was definitely established that a number of isonicotinyl hydrazones are more effective chemotherapeutic agents for tuberculosis than either streptomycin or isonicotinic acid hydrazide, which indeed has a very narrow margin of safety.

Table IV summarizes the results of our investigations and there the more active compounds only are listed.[1] From the table, it is apparent that the first nine most active antitubercular agents consist of the following isonicotinyl hydrazones (given in the order of their activity) derived from D-galacturonic acid, D-glucuronolactone, 2-hydroxy-5-chlorobenzaldehyde, salicylidene-acetone, 4-methoxybenzalacetone, or anisalacetone, 2-hydroxybenzaldehyde or salicylaldehyde, 3-hydroxybenzaldehyde, 4-methoxy-propiophenone, and 4-diethylaminobenzaldehyde. These agents, when given to sick mice infected with *Mycobacterium tuberculosis* H37Rv for a period of four to five weeks at a dosage of 0.05% in the diet, protected these mice from death and actually cured them of the disease; for the results of autopsy of these surviving animals revealed that they were like normal and showed no tuberculous infections in their internal organs. At a dosage of 0.025% in the diet, the activity of these agents was only very slightly decreased; the mortality rate was only 10% and the surviving animals showed no signs of infections in their organs after autopsy.

The second nine most active isonicotinyl hydrazones that followed very closely to those mentioned above are the derivatives from pyridoxal *beta*-ionone, 2-hydroxy-5-bromobenzaldehyde, 4 methoxy-acetophenone, 4-ethoxy-acetophenone, furfuralacetone, vanillalacetone, levulinic acid, and 4-dimethylaminobenzaldehyde. These agents, too, at a dosage of 0.05% in the diet given to sick mice for a period of four to five weeks, protected them completely from death and cured them from the disease. At a dosage of 0.025% in the diet, however, a considerable decrease of activity was seen; the mortality rate was 20%; the surviving mice, after autopsy, showed very mild though definite signs of infections in some of their internal organs of some of the animals (one or two of the surviving eight).

Streptomycin, given to the sick mice at a dosage of 3,000 units per week by subcutaneous injection, as a standard for comparison, did not protect them

[1] The *in vivo* antitubercular activity of a number of isonicotinyl hydrazones listed here had been confirmed independently by W. B. Sutton of the Lilly Research Laboratories, Indianapolis, Ind., and by Ben Freedlander of the Mount Zion Hospital, San Francisco, Calif. The authors wish to thank them for their kind collaboration in checking the important biological results.

TABLE IV.—IN VIVO ANTITUBERCULAR ACTIVITY OF ISONICOTINYL HYDRAZONES IN MICE INFECTED WITH MYCOBACTERIUM TUBERCULOSIS H37Rv

Compounds Tested	Dosage in Diet, %	(A)	(B)	(C)	(D)
D-Galacturonic acid isonicotinyl hydrazone	0.05	10	28	0	28+
	0.025	10	28	0	28+
D-Glucuronolactone INH (INHG)	0.05	10	28	0	28+
	0.025	10	28	10	27−
2-Hydroxybenzaldehyde INH (INHS)	0.05	10	35	0	35+
	0.025	10	35	10	30−
3-Hydroxybenzaldehyde INH	0.05	10	35	0	35+
	0.025	10	35	10	29−
4-Hydroxybenzaldehyde INH	0.1	10	35	0	35+
	0.05	10	35	20	28−
4-Methoxybenzaldehyde INH (INHA)	0.1	5	35	0	35+
	0.05	10	35	10	34−
4-Dimethylaminobenzaldehyde INH	0.05	5	35	0	35+
	0.025	10	35	20	28−
4-Diethylaminobenzaldehyde INH	0.05	5	35	0	35+
	0.025	10	35	10	32−
3-Methoxy-4-hydroxybenzaldehyde INH	0.1	5	33	0	33+
	0.05	10	33	20	28−
3-Ethoxy-4-hydroxybenzaldehyde INH	0.1	5	33	0	33+
	0.05	10	33	10	30−
Piperonal INH	0.1	5	33	0	33+
	0.05	10	33	20	27−
2-Hydroxy-5-chlorobenzaldehyde INH (INHCS)	0.05	5	33	0	33+
	0.025	10	33	10	31−
2-Hydroxy-5-bromobenzaldehyde INH (INHBS)	0.05	5	33	0	33+
	0.025	10	33	20	28−
4-Chlorobenzaldehyde INH	0.1	5	33	0	33+
	0.05	10	33	30	27−
4-Methoxy-acetophenone INH (INHMAP)	0.05	5	30	0	30+
	0.025	10	30	20	28−
4-Aminoacetophenone INH	0.05	5	30	0	30+
	0.025	10	30	20	27−
2-Hydroxy-acetophenone INH	0.1	5	30	0	30+
	0.05	10	30	10	29−
	0.025	10	30	30	26−
3-Hydroxy-acetophenone INH	0.1	5	30	0	30+
	0.05	10	30	20	28−
4-Hydroxy-acetophenone INH	0.1	5	30	0	30+
	0.05	10	30	20	27−
4-Chloro-acetophenone INH	0.1	5	30	0	30+
	0.05	10	30	30	25−
4-Ethoxy-acetophenone INH (INHEAP)	0.05	10	30	0	30+
	0.025	10	30	20	27−
4-Hydroxy-3-methoxy-acetophenone INH	0.1	5	30	0	30+
	0.05	10	30	30	26−
4-Methoxy-propiophenone INH (INHMPP)	0.05	5	31	0	31+
	0.025	10	31	10	30−
4-Hydroxy-propiophenone INH	0.1	5	31	0	31+
	0.05	10	31	10	30−
	0.025	10	31	30	25−
4-Aminopropiophenone INH	0.05	5	31	0	31+
	0.025	10	31	30	26−
4-Chloropropiophenone INH	0.1	5	31	0	31+
	0.05	5	31	20	28−
	0.025	10	31	40	20−
4-Methoxybenzalacetone INH or anisalacetone INH (INHAA)	0.05	5	31	0	31+
	0.025	10	31	10	30−
Vanillalacetone INH (INHVA)	0.05	5	31	0	31+
	0.025	10	31	20	28−
Furfuralacetone INH (INHFA)	0.05	5	35	0	35+
	0.025	10	35	20	30−
Salicylidene-acetone INH (INHSA)	0.05	10	35	0	35+
	0.025	10	35	10	33−
Benzalacetone INH	0.1	5	35	0	35+
	0.05	5	35	20	29−
α-Ionone INH	0.1	5	35	0	35+
	0.05	5	35	20	28−
	0.025	5	35	40	24−
β-Ionone INH (INHBI)	0.1	5	35	0	35+
	0.05	5	35	0	35+
	0.025	5	35	20	29−
Pyridoxal INH (INHPV)	0.1	5	35	0	35+
	0.05	5	35	0	35+
	0.025	5	35	20	30−
Levulinic acid INH	0.05	5	35	0	35+
	0.025	10	35	20	28−
4-Acetaminobenzaldehyde INH	0.1	5	35	0	35+
	0.05	5	35	20	28−
	0.025	10	35	40	25−
Benzaldehyde INH	0.1	5	35	0	35+
	0.05	10	35	20	28−
	0.025	5	35	40	25−
Cinnamaldehyde INH	0.1	5	35	0	35+
	0.05	10	35	10	30−
	0.025	5	35	40	25−
Cuminaldehyde INH	0.1	5	35	0	35+
	0.05	10	30	10	31−
	0.025	10	35	30	27−
Acetophenone INH	0.1	5	35	0	35+
	0.05	10	35	20	26−
	0.025	10	35	30	24−
Propiophenone INH	0.1	5	35	0	35+
	0.05	10	35	10	29−
	0.025	5	35	40	24−
Isonicotinic acid hydrazide (standard for comparison)	0.2[a]	10	35	50	20−[b]
	0.1	10	35	10	30−[b]
	0.05	10	35	0	35+
	0.025	10	35	20	31−
	0.01	5	35	40	23−
Streptomycin (standard)	3000 units weekly by s. c. inj.	60 or 6 groups of 10 each	28–35	20	27–32
Control	No drug	60 or 6 groups of 10 each	28–35	90–100	14–18

(A) = number of mice; (B) = days on test; (C) = % mortality; and (D) = mean survival time in days.

[a] The daily intake of the drug by the mouse weighing 15 Gm. was estimated to be about 120 mg./Kg.

[b] Animals died of drug toxicity (symptoms of convulsions) rather than of tuberculosis.

completely from death. The mortality rate was 20%. This was observed only when the experimental period lasted from four to five weeks. No death could be observed if the experiments were concluded at the end of two to three weeks. The surviving mice, on autopsy, showed mild but definite signs of infections in some of the internal organs in a number of the animals (three to four out of the surviving eight). Streptomycin, therefore, is definitely not so effective as the more active forms of isonicotinyl hydrazones mentioned in this paper.

Isonicotinic acid hydrazide, given to sick mice weighing between 15 and 16 Gm. for a period of thirty-five days at a high dosage of 0.2% in the diet (the daily intake of the drug was estimated to be about 120 mg./Kg.), killed 50% of the mice tested. Before dying, these animals showed signs of nervousness, light convulsions, and other toxic symptoms characteristic of the drug. The results of autopsy after their death showed marked tuberculous infections at some internal organs (lungs, livers, and hearts), which were absent from those mice that

survived after thirty-five days. These results seemed to indicate that (*a*) the LD_{50} for sick mice could be much lower than that for normal mice [about 165 mg./Kg. orally according to Pan and co-workers (42)] and (*b*) the effectiveness of a potent drug is not necessarily directly proportional to the dosage; if given at too high a dosage, the animals might become doubly sick (sick of the disease and also sick of the drug toxicity) and this double sickness could kill the animal before the maximum drug-effectiveness was reached.

At a dosage of 0.1% in the diet, isonicotinic acid hydrazide seemed to exert its maximum chemotherapeutic action; the mortality rate of the sick animals was only 10% but the results of autopsy of all the ten animals treated with the drug showed no signs of tuberculous infections at any of the internal organs. The one mouse which died, died probably of the accumulative toxic effect of the drug. The daily intake of the drug was estimated to be between 60 and 65 mg./Kg. mice which is about the maximum tolerated dose of the drug. At a dosage of 0.05% in the diet, the mice were all protected from death during the experimental period of thirty-five days. The autopsy of the surviving animals, however, revealed that there were mild tuberculous infections in some of the internal organs, indicating that at a dosage of 0.05% in the diet, the disease was merely well suppressed and not completely cured by isonicotinic acid hydrazide. Dosages below that of 0.05% were found to be definitely inefficient; the mortality rate increased and the autopsy results revealed more frequent and more severe infections in the internal organs of the dead as well as the surviving mice.

With the exceptions of (*a*) D-glucuronolactone isonicotinyl hydrazone, which was already proved to be an excellent antitubercular agent, in the clinics on human patients, by Dr. Cabot Brown, *et al.* (39), and independently by French physicians in Paris (40), and of (*b*) D-galacturonic acid isonicotinyl hydrazone, the low toxicity of which had been established in this laboratory (38, 41), the hope of the other isonicotinyl hydrazones of becoming top chemotherapeutic agents for tuberculosis and perhaps leprosy will depend largely on their acute as well as chronic toxicity in animals before they can be introduced into the hospitals for clinical trials on human patients.

Preliminary acute toxicity studies on some of the most active forms of isonicotinyl hydrazones, namely, those derived from salicylaldehyde, 2-hydroxy-5-chlorobenzaldehyde, 2-hydroxy-5-bromobenzaldehyde, and 4-methoxybenzalacetone or anisalacetone, seemed to indicate that mice could tolerate more than 3 Gm./Kg. of these new agents orally without showing any symptom of toxicity. Research is still in progress and results will be published as soon as they are complete.

SUMMARY

1. Three simple, general procedures for the preparation of isonicotinyl hydrazones have been described.

2. With these procedures, 106 aldehydes and ketones have been tested with the reagent, isonicotinic acid hydrazide and 93 of them have yielded crystalline isonicotinyl hydrazones, suitable for the purpose of identification.

3. Isonicotinyl hydrazones which crystallize only slowly may be converted into their hydrochlorides and identified.

4. A large number of isonicotinyl hydrazones have been found to possess *in vivo* activity against *Mycobacterium tuberculosis* H37Rv higher than that of streptomycin and at least of the same order of magnitude as isonicotinic acid hydrazide.

5. Toxicological and pharmacological studies of these isonicotinyl hydrazones are still in progress. The results will be published as soon as the investigation is complete.

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Investigation of Some Complexes Formed in Solution By Caffeine*

V. Interactions Between Caffeine and *p*-Aminobenzoic Acid, *m*-Hydroxybenzoic Acid, Picric Acid, *o*-Phthalic Acid, Suberic Acid, and Valeric Acid

By TAKERU HIGUCHI and JOHN L. LACH

Additional experimental data on the complexing behavior of caffeine with organic compounds are presented. The relative complexing tendencies and the stoichiometric relationships of caffeine with a number of organic acids have been evaluated. It has been shown by means of phase studies that the stability constants of these complexes are in the order of *p*-aminobenzoic acid ≃ *m*-hydroxybenzoic acid > *o*-phthalic acid > picric acid > suberic acid > valeric acid = 0.

IN EARLIER PAPERS from these laboratories (1–4) experimental evidence for the existence and the nature of a large number of complexes formed by caffeine with pharmaceutical compounds were discussed. In this communication the results of further investigation on the complexing tendencies of the xanthine with a number of organic acids are presented. New data are reported for interactions with two aliphatic acids, suberic and valeric acid, and two aromatic acids, *o*-phthalic and picric acid. Some additional data on caffeine-*p*-aminobenzoic acid and caffeine-*m*-hydroxybenzoic acid systems are also offered.

EXPERIMENTAL OBSERVATIONS ON THE ORGANIC ACIDS

The extent of complex formation between caffeine and the acids was studied in most part by the solubility method. As previously pointed out (5) the method is quite well suited to studies of this nature since isolation of unstable reaction products is not essential. Stoichiometric relationships of the complexing reaction can be calculated from phase diagrams obtained from these solubility studies.

***p*-Aminobenzoic Acid Interaction.**—The caffeine-*p*-aminobenzoic acid system has already been given some attention, an earlier study having shown that a 1:1 complex was formed. There was some evidence that at higher caffeine concentration another complex existed and was responsible for the solubilization of the first complex. The study of this second interaction was extended in the present investigation to obtain additional information as to the stoichiometric relationship in the region of higher caffeine concentration.

The results of the present study on the effect of higher caffeine concentration on the apparent solubility of *p*-aminobenzoic acid are shown in Fig. 1. The stoichiometric and equilibrium relationship derived from the left-hand portion of the diagram has already been discussed (4) and will not be taken up in detail at the present time. It suffices to say that the experimental results are in accord with the assumption that the complexing reaction

$$\text{PABA} + \text{Caffeine} \rightleftharpoons \text{PABA-Caffeine} \qquad (1)$$

takes place in the system. According to this reasoning, the solubility of the initial complex (1:1)

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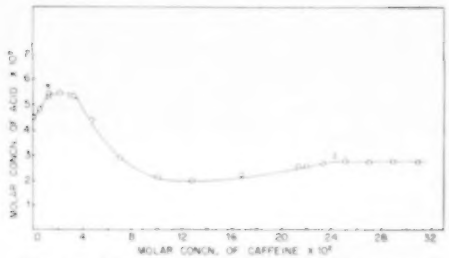

Fig. 1.—Phase diagram of PABA-Caffeine system in water at 30° C. Molar concn. of solubilized *p*-aminobenzoic acid plotted against molar concn. of caffeine.

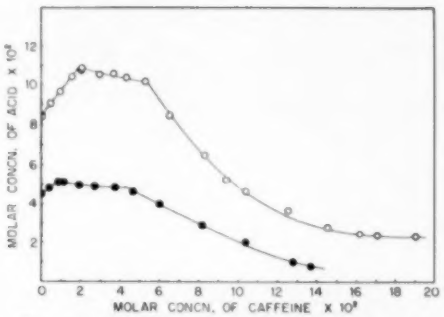

Fig. 2.—Solubilizing action of caffeine on the apparent solubility of *m*-hydroxybenzoic acid. ○—30°, ●—15°.

was 1.0×10^{-2} moles/liter at 30°.[1] Actually at higher caffeine concentration such that most of the acid would be expected to be in the complexed form, the equilibrium concentration of the acid in solution was considerably higher than that of the solubility of the 1:1 complex. This behavior indicated the existence of a second complex which formed in an increasing amount until a limiting value was reached as shown by the plateau region at point *Z*. This break into a plateau was due to the saturation of the solution with respect to caffeine and not to the precipitation of the new complex. In a manner analogous to the benzocaine-caffeine interaction it can be shown that the stoichiometric ratio of the complex formed at higher caffeine concentrations was 1 acid molecule to 3 caffeine molecules.

***m*-Hydroxybenzoic Acid Interaction.**—Although the *m*-hydroxybenzoic acid-caffeine interaction has been previously studied in a preliminary fashion by Higuchi and Zuck (3) they were unable to arrive at any definite stoichiometric relationship for this interaction. The present study has shown that this was due in part to a tendency of the formed complexes to supersaturate readily at 15°. Reproducible results were obtained only on seeding of the solutions after several hours of equilibration. Results obtained based on this technique are plotted in Fig. 2 showing the behavior of *m*-hydroxybenzoic acid in the presence of caffeine in water at 15° and 30°.

At low caffeine concentrations a straight line relationship between the acid in solution and the added caffeine was obtained. The slope of the line, however, was greater than one and was indicative of higher complex formation, possibly 2 acid molecules to 1 caffeine molecule, in addition to the 1:1 complex as represented by equations 2 and 3. Un-

$$\text{Acid} + \text{Caffeine} \rightleftharpoons \text{Acid-Caffeine} \qquad (2)$$
$$\text{Acid} + \text{Acid-Caffeine} \rightleftharpoons \text{Acid}_2\text{-Caffeine} \qquad (3)$$

like the plateau found in the PABA-caffeine system, the region corresponding to the precipitation of the complexes is curved, a behavior probably ascribable to coprecipitation of higher complexes with the 1:1 species. Although more than one type of complex is indicated in the system it appeared that at higher caffeine concentrations the principal precipitated form was the one in which the ratio was 1:1 with respect to the acid and caffeine. The phase diagram obtained at 30° is apparently in accord with this 1:1 relationship. At 15°, however, the results obtained from the diagram were more in agreement with a 2:1 type of a complex, i. e., 2 acid molecules to 1 caffeine molecule. Formation of this type of complex at lower temperature can be partially rationalized on the basis that at 30° it is too soluble to permit isolation. From preliminary studies of this interaction at 15° (3) it was shown that the complex, or complexes, stable to recrystallization was the one in which the ratio was 1:1 with respect to the acid and caffeine. It is evident from the information obtained at the two temperatures that the over-all reaction is of a highly complex nature.

Picric Acid Interaction.—The results obtained, as shown in Fig. 3, of the solubility behavior of picric acid in presence of caffeine are similar to those of the hydroxybenzoic acids. The complex formed in this system appeared to be extremely insoluble in nature since the initial increase in the apparent solubility of picric acid was small as is evident from the diagram. Analogous to the PABA-caffeine system, formation of a complex with caffeine content greater than one was being formed at higher caffeine concentrations as shown by the increase in the amount of limiting acidic material in solution. Calculation of the component ratio entering into complex formation in the plateau region of the phase diagram, however, gave a 1:1 relationship. Results of a chemical analysis of the complex, m. p. 143–145°, corresponded to this composition.

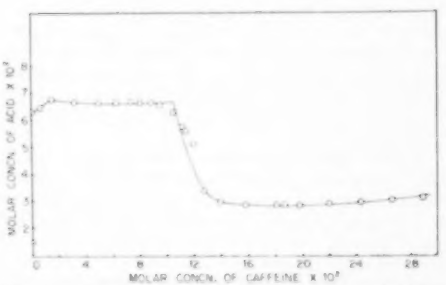

Fig. 3.—Phase diagram showing the solubility behavior of picric acid in presence of caffeine.

[1] The difference between the solubility limit of the complex, point *R* in Fig. 1, and the solubility of PABA at zero caffeine concentration is equal to the solubility of the 1:1 complex.

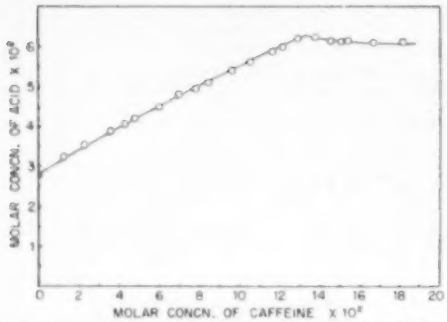

Fig. 4.—Influence of different amounts of caffeine on the solubility of *o*-phthalic acid.

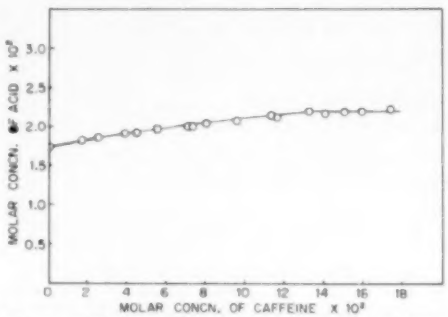

Fig. 5.—Effect of caffeine on the apparent solubility of suberic acid.

o-Phthalic Interaction.—The phase diagram showing the interaction between *o*-phthalic acid and caffeine at 30° is given in Fig. 4. The increase in amount of acid in solution caused by complex formation on addition of caffeine continued until a limiting value was reached as represented by the flat portion of the phase diagram. The formation of this region was not due to precipitation of the complexes as was the case in the hydroxybenzoic acid systems but rather to the limited solubility of caffeine itself, the solution being saturated with respect to the xanthine instead of the complex. At this point the solid phase in the system contained solid caffeine and solid acid. Stoichiometric relationships of the complexing reaction calculated from the increases in solubilities of both the acid and caffeine in a manner analogous to the benzocaine-caffeine interaction (4) gave a 1:2 ratio with respect to *o*-phthalic acid and caffeine. The stability constant calculated on this basis, however, did not yield a constant value, a better agreement being obtained on the basis of a 1:1 type of interaction. This apparent discrepancy may be partially attributed to the fact that caffeine can exist as a dimer in the aqueous phase (1). Further studies are necessary, however, to elucidate the mechanism of this unusual behavior.

Suberic Acid Interaction.—The effect of varying caffeine concentration on the apparent solubility of suberic acid is shown in Fig. 5. It is seen from the phase diagram that the increase in solubility is small even at relatively high caffeine concentration. The slight reaction tendency is not altogether unexpected. The absence of an aromatic group in the molecule, which in itself favors complex formation, can lead to such behavior. It is difficult to calculate the exact stoichiometry of the interaction from the phase diagram; however, it is likely that the complex is of the 1:1 type since this type seems to be the dominant one in studies of the acid caffeine interactions.

Valeric Acid Interaction.—The partition coefficient method previously described for the caffeine-benzoic acid interaction (1) was employed in the study of the valeric acid-caffeine system since the liquid form of valeric acid did not permit the use of the solubility procedure. The aliphatic acid when studied in this manner, however, showed no tendency to undergo complex formation with the xanthine.

GENERAL DISCUSSION

The results of the present investigation give additional evidence as to the prevalence of this type of an interaction. For the acids studied the extent and tendency to complex appear to be approximately in the order of *p*-aminobenzoic acid ≅ *m*-hydroxybenzoic acid > *o*-phthalic acid > picric acid > suberic acid > valeric acid = 0. Estimated values of the stability constants are listed in Table I.

TABLE I.—ESTIMATED VALUES OF THE STABILITY CONSTANTS

Acid	Stability Constant
p-Aminobenzoic	48
m-Hydroxybenzoic	[a]
o-Phthalic	14[b]
Picric	8
Suberic	3
Valeric	0

[a] Indeterminate but substantial.
[b] Calculated on a 1:1 basis.

EXPERIMENTAL PROCEDURE

Reagents.—Recrystallized *m*-hydroxybenzoic acid, m. p. 201–202°; *p*-aminobenzoic acid, m. p. 187–188°; picric acid, m. p. 123–124°; *o*-phthalic acid, m. p. 205–207°; suberic acid, m. p. 140–141°; 0.05 *N* $Ba(OH)_2$ solution; phenolphthalein, brom cresol green, and brom thymol blue as indicators.

Procedure.—Excess quantities of the acid were placed in 125-ml. glass-stoppered bottles together with varying amounts of caffeine and 50 ml. of solvent added to each. The bottles were placed in a mechanical shaker in a constant temperature bath and equilibrated for a number of hours. Aliquot portions of the supernatant liquid were removed and analyzed for the acid content by titration with barium hydroxide solution. The amount of acid, volume of solvent, equilibration time, and indicators used were as follows:

p-Aminobenzoic acid; 0.5 Gm./50 ml. water; twenty-four hours; brom thymol blue.

m-Hydroxybenzoic acid; 1.0 Gm./50 ml. sulfuric acid solution; six hours; brom thymol blue.

Picric acid; 0.9 Gm./25 ml. water; fourteen hours; brom cresol green.

o-Phthalic acid; 1.0 Gm./50 ml. sulfuric acid solution; six hours; phenolphthalein.

Suberic acid; 0.25 Gm./50 ml. sulfuric acid solution; six hours; phenolphthalein.

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Investigation of Complexes Formed in Solution by Caffeine*

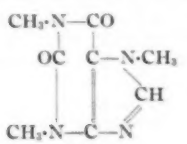
VI. Comparison of Complexing Behaviors of Methylated Xanthines with *p*-Aminobenzoic Acid, Salicylic Acid, Acetylsalicylic Acid, and *p*-Hydroxybenzoic Acid

By TAKERU HIGUCHI and JOHN L. LACH

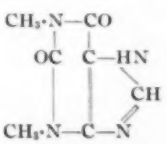
Previous studies have shown that caffeine forms molecular complexes in solution with various compounds. The present report extends this investigation to other methylated xanthines. It has been shown that, except for the *p*-aminobenzoic acid interaction, caffeine has the greatest solubilizing action on the acids listed in the title while theobromine exhibits the smallest effect. The results also indicate that the solubility of the complexes formed parallels the solubility of the parent xanthines. Although their solubilities differ greatly, the stability constants of the complexes formed with any given acid by the three xanthines are usually quite similar.

ALTHOUGH methylated xanthines are known to form complexes with many pharmaceutical compounds, only those of caffeine have been studied extensively (1–5). The results of investigation of the complexing tendencies of two other xanthines, theophylline and theobromine, with a number of organic acids, are presented in this communication and are compared with similar data obtained earlier on caffeine.

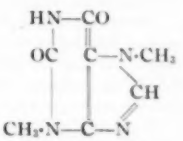
Although the molecular structure of the three xanthines as shown below possess more than superficial resemblance, the intermolecular binding tendencies of these compounds are significantly different as reflected in their physical constants listed in Table I.



Caffeine



Theophylline



Theobromine

TABLE I.—SOME PHYSICAL CONSTANTS OF THE METHYLATED XANTHINES

Xanthine	Molecular weight	M. P.	Solubility moles/liter × 10^3 at 30°
Caffeine	194.19	238	13.3
Theophylline	180.17	270–274	4.5
Theobromine	180.17	357	≃0.3

The dimethylated compounds have higher melting points and lower solubilities than caffeine, indicating stronger mutual binding in their respective solid states. The absence of the extra methyl group in theophylline and theobromine probably permits formation of stronger dipole-dipole bonds in these substances as compared to caffeine. It seems probable that these same differences would also carry over in their complexing tendencies.

Experimental procedures employed in these studies were essentially those described in con-

* Received February 18, 1954, from the School of Pharmacy, University of Wisconsin, Madison.

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Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

nection with the caffeine investigation and are not reproduced in detail here. The method is based on solubility study of the reacting components and is particularly suitable for detecting small complexing tendencies.

EXPERIMENTAL OBSERVATIONS

Interactions with *p*-Aminobenzoic Acid.—The effects of varying theophylline and theobromine concentrations on the solubility behavior of *p*-aminobenzoic acid[1] in water at 30° are shown in Fig. 1 along with earlier data reported for the caffeine interactions (4, 5). The quantities of acid per 50 ml. water used in these solubility studies were 0.5 Gm. for caffeine, 1.0 Gm. for theophylline, and 0.6 Gm. for theobromine.

From the phase diagrams obtained it is seen that all three xanthines underwent definite complexing reactions. On comparison of the theophylline interaction with that of caffeine the chief difference appeared to be in the solubility of the initial complex. Analysis of the phase diagram, in a manner described for the caffeine interactions, and chemical analysis of the precipitated reaction product gave a 1:1 molecular ratio for the theophylline complex similar to that of caffeine. The stability constants calculated from the straight line relationships obtained before saturation of the solution with respect to the complex gave average values of 48 and 31 for caffeine and theophylline, respectively. Although the theophylline complex is considerably more soluble, it is less stable as is evident from these values. The theophylline diagram was rather difficult to obtain. Reproducible results for the system were possible only after seeding since the complex formed supersaturated readily at 30°.

The plateau region in the theophylline system, unlike that found in the caffeine interaction, was curved. This behavior may be attributed in part to a coprecipitation of the higher complexes with the 1:1 type and partly to a residual amount of complexes still present in solution due to incomplete precipitation after seeding. This plateau corresponds to the region of complex precipitation and is dependent on the amount of excess solid acid present in the system. The formation of more than one complex is evidenced by the amount of acidic material remaining in solution at higher xanthine concentrations as compared to the indicated solubility of the initial complex. Although the studies were not carried out to saturation with respect to theophylline, it is likely that a situation similar to that found in the caffeine system may exist at higher theophylline concentrations. In a previous report (5) it was shown that at higher caffeine concentrations a second complex, with caffeine content greater than one, was formed.

Theobromine apparently interacts to only a small extent with the acid as is evident in Fig. 1. The break in the diagram to a plateau region was due apparently to the insoluble nature of theobromine, the system having become saturated with respect to the xanthine and not to the complex.

Interactions with Salicylic Acid.—In Fig. 2 is shown the influence of theophylline and theobromine on the apparent solubility of salicylic acid[2] in water at 30°. The results from an earlier study (3) of the caffeine-salicylic acid system are also included in the figure for comparison purposes. The quantities of acid per 50 ml. water used were 0.35 Gm. for the caffeine study and 0.4 Gm. for theophylline and theobromine.

From the phase diagrams obtained it is evident that the three xanthines reacted similarly. Analysis of the diagrams and chemical analysis of the precipitated reaction products all gave 1:1 relationships with respect to the acid and the corresponding xanthine. Because of the low solubility of both the theophylline complex and the theobromine complex it was difficult to calculate the stability constants for these interactions from the straight line relationships obtained at low xanthine concentrations. On the basis of this 1:1 relationship, however, determination of these constants from the slopes of the lines obtained to the right of the plateau regions of the phase diagrams indicated that they were of the same order of magnitude, having an approximate value of 40. From the results obtained it appears that the three xanthines undergo

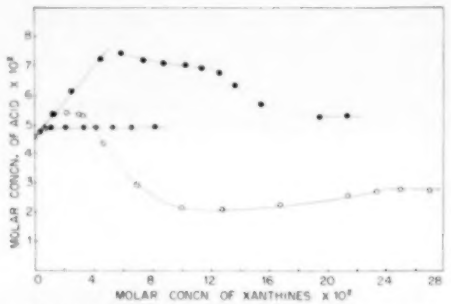

Fig. 1.—Phase diagram showing the interaction of *p*-aminobenzoic acid with several methylated xanthines in water at 30° C. ○—caffeine, ●—theophylline, ⊗—theobromine.

[1] The solubility of *p*-aminobenzoic acid per 50 ml. water at 30° is 0.311 Gm.

[2] The solubility of salicylic acid per 50 ml. water at 30° is 0.124 Gm.

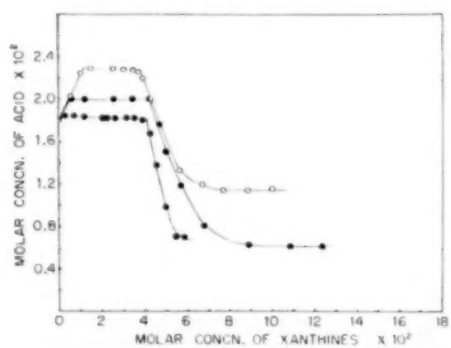

Fig. 2.—Phase diagram showing the solubility behavior of salicylic acid in presence of methylated xanthines in water at 30° C. ○—caffeine, ●—theophylline, ⊗—theobromine.

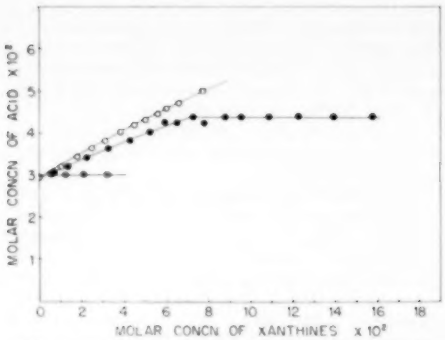

Fig. 3.—Phase diagram showing the interaction of aspirin with several methylated xanthines in water at 30° C. ○—caffeine, ●—theophylline, ⊗—theobromine.

a similar reaction with salicylic acid, the only apparent difference being in the solubility of the initial complex formed.

Interactions with Acetylsalicylic Acid.—The solubilizing action of theophylline and theobromine on aspirin[3] in water at 30° is given in Fig. 3. Included in this diagram, for comparative reasons, are the results previously reported by Higuchi and Zuck (3) for the caffeine-aspirin interaction. The quantities of acid used in these solubility studies per 50 ml. of water was 0.5 Gm. for the caffeine system and 0.6 Gm. for the other two xanthines.

From the phase diagrams obtained it is seen that of the three xanthines studied caffeine has the greatest solubilizing action on aspirin as is evident from the slopes of the lines. Although the caffeine interaction was not studied to saturation of the solution with respect to caffeine, a plateau region as that found in the theophylline system would be expected at higher concentrations of the xanthine. This region, as previously pointed out (4), corresponds to saturation of the solution with respect to the xanthine and not to precipitation of the complexes. Invariant stability constants with average values of 14.7 for the caffeine system and 8.5 for the theophylline system were obtained from the straight line portions of the phase diagrams when calculated on a 1:1 basis. Some doubt is cast on the significance of these calculations, however, by the fact that on analysis of the phase diagram for the theophylline interaction a stoichiometric ratio of 1:2 with respect to the acid and the xanthine was obtained. An attempt to calculate the constant on this basis, on the other hand, gave inconsistent values. No explanation of this behavior is presented at this time.

Accurate determination of the constant for the theobromine interaction was impossible because of the very low solubility of theobromine. It appeared, however, that the order of magnitude of this constant was comparable to that of the theophylline system.

Interactions with *p*-Hydroxybenzoic Acid.—The effects of theophylline and theobromine on the equilibrium amount of *p*-hydroxybenzoic[4] acid in solution at 30° are shown in Fig. 4 in addition to data obtained earlier for the caffeine interaction (3). The amounts of acid per 50 ml. water employed in these studies were 1.0 Gm. for caffeine, 1.2 Gm. for theophylline, and 0.7 Gm. for theobromine.

From the results obtained it is evident that the phase diagrams for theophylline and theobromine are analogous to those of caffeine with other hydroxybenzoic acids (3). Although the caffeine interaction with *p*-hydroxybenzoic acid (3) was unfortunately limited to studies at relatively low caffeine concentrations, it is probable that a similar type of diagram would have resulted at higher concentrations of these xanthines. This caffeine interaction has been assumed to be of the 1:1 type, the stability constant calculated on this basis being relatively high since little free caffeine remained in solution. Although the constant was not precisely evaluated it was believed to be greater than 100.

The relatively small increase in the solubility of the acid produced by theophylline can be ascribed to the insoluble nature of the complex formed. Phase diagram analysis of this system gave a stoichiometric ratio of 1:1 with respect to the acid and theophylline. Results obtained on chemical analysis of the precipitated reaction product also corresponded to this composition. The stability constant calculated on this basis was approximately 14.

Although the theobromine-*p*-hydroxybenzoic acid interaction appeared to be analogous to the theophylline system, analysis of the diagram and chemical analysis of the precipitated complex gave a 2:1 ratio with respect to the acid and xanthine. It is apparent that the nature of this reaction differs from that of theophylline and of caffeine.

Although a direct comparison of the stability constants of the complexes formed by the three xanthines is not possible because of qualitative differences, it is seen from the results obtained that caffeine exerts the greatest solubilizing action on the acid.

GENERAL DISCUSSION

From the results obtained it appears that the complexing interactions of the two other methylated xanthines, theophylline and theobromine, are quali-

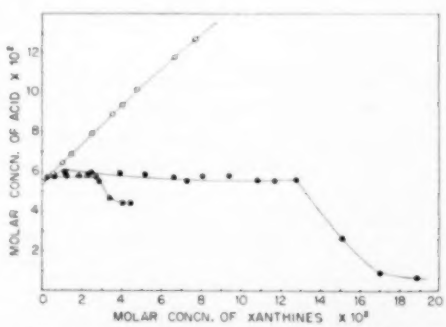

Fig. 4.—Phase diagram showing the effect of several methylated xanthines on the apparent solubility of *p*-hydroxybenzoic acid in water at 30° C. ○—caffeine, ●—theophylline, ⊗—theobromine.

[3] The solubility of aspirin in water per 50 ml. at 30° is 0.264 Gm.

[4] The solubility of *p*-hydroxybenzoic acid per 50 ml. water at 30° is 0.379 Gm.

tatively very similar to those of caffeine. Evidence indicates that, except for the theophylline-*p*-aminobenzoic acid complex, the solubilities of the products formed in these interactions parallel the solubilities of the parent xanthines. This suggests that the nature of the complexes are such that the groups bound to the xanthine molecules have only secondary masking effect on the forces which normally bind these molecules together in their solid state.

EXPERIMENTAL PROCEDURE

Reagents.—Recrystallized theophylline, m. p. 272–273°; theobromine, m. p. 357°; *p*-aminobenzoic acid, m. p. 187–188°; salicylic acid, m. p. 158°; acetylsalicylic acid, m. p. 135°; *p*-hydroxybenzoic acid, m. p. 214–216°; 0.05 *N* barium hydroxide solution; brom thymol blue; rosalic acid; methyl red, and chlorphenol red as indicators.

Procedure.—The experimental procedure and method of analysis employed in these studies were identical with those for the caffeine interactions (4, 5) except for the following differences: A weakly acidic (0.003 *N*) sulfuric acid solution was employed as the solvent for salicylic acid, *p*-hydroxybenzoic acid, and aspirin systems, the acidic solution being used to suppress any dissociation of the acids. Distilled water was the solvent for the *p*-aminobenzoic acid interaction. Equilibration time for these systems at 30° was fourteen hours except for aspirin, for which the time was three hours.

The quantities of acid per 50 ml. solvent and indicators used for theophylline and theobromine reactions were as follows:

Theophylline.—*p*-Aminobenzoic acid (1.0 Gm.); rosalic acid, salicylic acid (0.4 Gm.); methyl red, aspirin (0.6 Gm.); methyl red, *p*-hydroxybenzoic acid (1.2 Gm.); methyl red.

Theobromine.—*p*-Aminobenzoic acid (0.6 Gm.); brom thymol blue, salicylic acid (0.4 Gm.); chlorphenol red, aspirin (0.6 Gm.); chlorphenol red, *p*-hydroxybenzoic acid (0.7 Gm.); brom thymol blue.

REFERENCES

(1) Higuchi, T., and Zuck, D., THIS JOURNAL, **41**, 10 (1951).
(2) *Ibid.*, **42**, 132(1953).
(3) *Ibid.*, **42**, 138(1953).
(4) Higuchi, T., and Lach, J. L., THIS JOURNAL, **43**, 349 (1954).
(5) *Ibid.*, **43**, 524(1954).

The Kinetics of Degradation of Chloramphenicol in Solution*

III. The Nature, Specific Hydrogen Ion Catalysis, and Temperature Dependencies of the Degradative Reactions

By TAKERU HIGUCHI and ARNOLD D. MARCUS

The degradation of chloramphenicol in aqueous solutions below *p*H 7 has been shown to occur virtually entirely through hydrolytic cleavage of the amide function. It has been demonstrated that specific hydrogen-ion catalyzed hydrolysis plays a major role in the degradation of the antibiotic in aqueous solutions below *p*H 2. The temperature dependencies of the uncatalyzed (water) reaction and hydrogen-ion catalyzed reaction have been determined and the catalytic constants for the various reactions contributing to the over-all degradative rate have been evaluated. A possible mechanism to account for hydrogen-ion catalyzed hydrolysis is also presented.

IN A PREVIOUS communication (1) it was reported that chloramphenicol was subject to a type of general acid-base catalyzed degradation. These studies also showed that an uncatalyzed reaction contributed significantly to the over-all degradative rate. The results of a further study establishing the chemical nature of the breakdown reaction, evaluating the heats of activation of the several reactions, and demonstrating specific hydrogen-ion catalysis during the degradative step are presented at this time.

The experimental procedures employed to effect degradation of the antibiotic and the analytical methods used have been reported previously (1, 5, 7) and are not repeated here.

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Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
This project was supported in part by the Research Committee of the Graduate School with funds supplied by the Wisconsin Alumni Research Foundation.

It was only after completion of this manuscript that the recent work of Trolle-Lassen [*Arch. Pharm. Chemi*, **60**, 689(1953)] became available in this country. In all humility the present authors would like to express their esteem for Dr. Trolle-Lassen's work. In general our findings are in excellent agreement with his results. The principal additional contribution attributable to the present study appears to be in the greater experimental detail. Because of the limited number of experimental runs made by Dr. Trolle-Lassen, his findings, for example, indicate no region of *p*H independence for the breakdown reaction whereas actually the rate is substantially constant from *p*H 2 to 6. The nature of the acid-catalyzed reaction, furthermore, becomes clearer as a result of the present investigation.

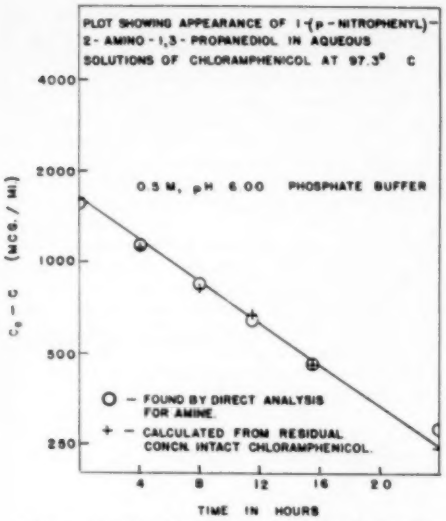

PLOT SHOWING APPEARANCE OF 1-(p-NITROPHENYL)-2-AMINO-1,3-PROPANEDIOL IN AQUEOUS SOLUTIONS OF CHLORAMPHENICOL AT 97.3° C
0.5 M, pH 6.00 PHOSPHATE BUFFER
O - FOUND BY DIRECT ANALYSIS FOR AMINE.
+ - CALCULATED FROM RESIDUAL CONCN. INTACT CHLORAMPHENICOL.
$C_0 - C$ (MCG./ML)
TIME IN HOURS

Fig. 1.—In this plot, the increase in the concentration of the amine base of chloramphenicol resulting from the degradation of the antibiotic has been plotted against time. To obtain a linear relationship, $C_0 - C$ has been plotted where C_0 was the concentration of the amine at equilibrium and C the concentration at any time t. It is evident that the results based on direct analysis are the same as those based on the assumption that degradation of one molecule of chloramphenicol yields one molecule of the amine.

ESTABLISHMENT OF THE NATURE OF THE DEGRADATIVE STEP

Previous experimental evidences (1, 2) strongly indicated that amide cleavage represented the principal route of degradation. Furthermore, it had been shown that one of the products of chloramphenicol metabolism was the corresponding amine 1-(*p*-nitrophenyl)-2-amino-1,3-propanediol (3) the same compound being produced by the reaction between chloramphenicol and strong acids or bases (3). These studies did not, however, entirely eliminate the possibility that other significant degradative pathways may exist. Indeed, it has been shown that reduction of the nitro group takes place in the presence of certain bacterial extracts (4) and that the antibiotic may be oxidized to *p*-nitrobenzaldehyde (3).

The extent of any secondary degradative reaction can be readily established by analyzing solutions of chloramphenicol at various stages of degradation for the residual drug and for the amine released by hydrolytic cleavage of the amide group. Any difference between the concentration of amine found by direct analysis and that calculated from the quantity of chloramphenicol which has undergone degradation can be attributed to some other degradative pathway. We have carried out this comparison for aqueous solutions of chloramphenicol at *p*H 6.00 and 4.65 utilizing an analytical procedure reported elsewhere (5).

As shown in Figs. 1 and 2, the rate of loss of the antibiotic from solution and the rate of appearance

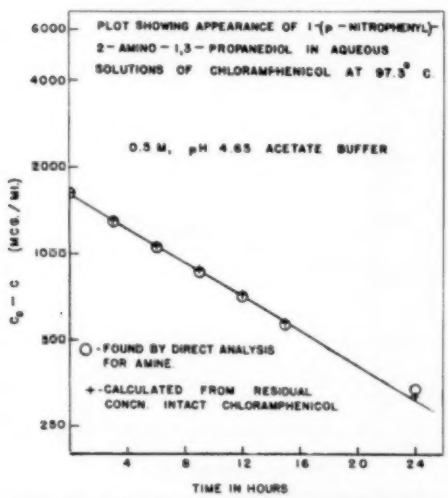

PLOT SHOWING APPEARANCE OF 1-(p-NITROPHENYL)-2-AMINO-1,3-PROPANEDIOL IN AQUEOUS SOLUTIONS OF CHLORAMPHENICOL AT 97.3° C.
0.5 M, pH 4.65 ACETATE BUFFER
O - FOUND BY DIRECT ANALYSIS FOR AMINE.
+ - CALCULATED FROM RESIDUAL CONCN. INTACT CHLORAMPHENICOL
$C_0 - C$ (MCG./ML)
TIME IN HOURS

Fig. 2.—This plot is similar to that of Fig. 1, except that the run was made at a much higher hydrogen-ion concentration.

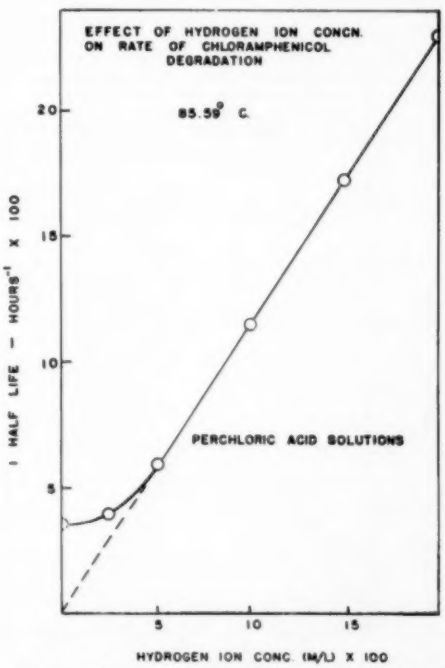

EFFECT OF HYDROGEN ION CONCN. ON RATE OF CHLORAMPHENICOL DEGRADATION
85.59° C.
PERCHLORIC ACID SOLUTIONS
$t_{\frac{1}{2}}$ HALF LIFE - HOURS^{-1} X 100
HYDROGEN ION CONC. (M/L) X 100

Fig. 3.—A plot showing the catalytic activity of hydrogen ions obtained from perchloric acid solutions upon the rate of hydrolysis of chloramphenicol at its amide linkage at 85.59° C. The broken circle does not represent an experimentally determined point, but was obtained by extrapolation to zero buffer concentration at the same temperature.

of the amine are essentially the same.[1] The points calculated from determination of chloramphenicol based upon the assumption that one amine molecule is formed for every molecule of the antibiotic lost are shown in both plots. It is evident that for all practical purposes the reaction involving amide cleavage was responsible for the entire observed loss of the antibiotic.

In view of the conclusions presented above and the known response of amides to high concentrations of hydrogen ions, there can be very little doubt that in aqueous solutions below *p*H 7, hydrolytic cleavage of the amide group represents the only significant route of chloramphenicol degradation.

SPECIFIC HYDROGEN ION CATALYSIS

It was shown previously (1) that chloramphenicol degradation is independent of hydrogen-ion concentration within the *p*H interval 2.3–6. Nevertheless there were reasons to believe that specific hydrogen-ion catalysis would occur at very low *p*H values. Earlier investigations into amide hydrolysis showed that the hydrolytic reaction exhibited no significant range of *p*H independence (6). Every previous investigation of the kinetics of amide hydrolysis indicated that hydrolytic cleavage was remarkably susceptible to catalysis by hydrogen ions (specific hydrogen-ion catalysis). It has been shown, more-

[1] The concentration of a product of any first order reaction is governed by the expression

$$-\ln (C_\infty - C_t) = kt + k'$$

where C_∞ is the concentration of the product at infinite time and C_t is the concentration at time t.

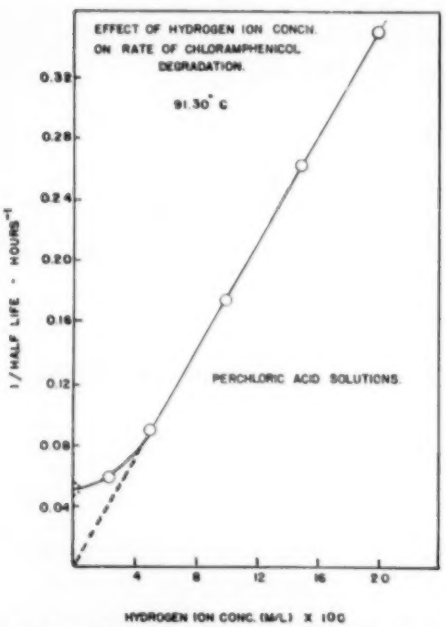

EFFECT OF HYDROGEN ION CONCN. ON RATE OF CHLORAMPHENICOL DEGRADATION.
91.30° C
PERCHLORIC ACID SOLUTIONS
1/HALF LIFE - HOURS⁻¹
HYDROGEN ION CONC. (M/L) X 100

Fig. 4.—A plot showing the catalytic activity of hydrogen ions upon the rate of hydrolytic degradation of chloramphenicol at 91.30° C. The broken circle does not represent an experimentally determined point, but was obtained by extrapolation to zero buffer concentration at the same temperature.

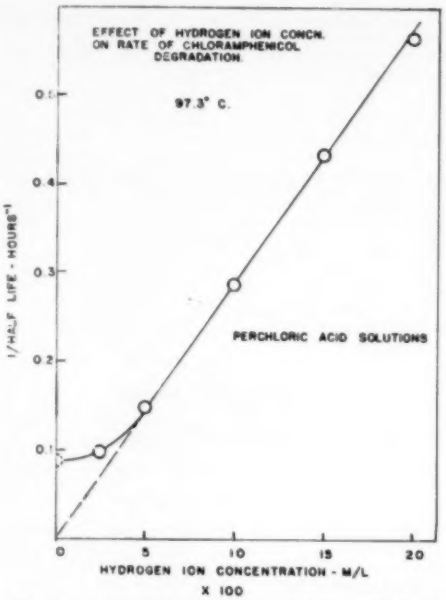

EFFECT OF HYDROGEN ION CONCN. ON RATE OF CHLORAMPHENICOL DEGRADATION.
97.3° C.
PERCHLORIC ACID SOLUTIONS
1/HALF LIFE - HOURS⁻¹
HYDROGEN ION CONCENTRATION - M/L X 100

Fig. 5.—A plot showing the catalytic activity of hydrogen ions upon the rate of hydrolytic degradation of chloramphenicol at 97.3° C. The broken circle does not represent an experimentally determined point, but was obtained by extrapolation to zero buffer concentration at the same temperature.

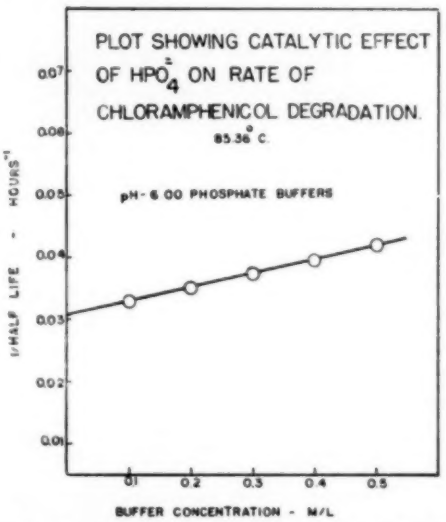

PLOT SHOWING CATALYTIC EFFECT OF HPO4= ON RATE OF CHLORAMPHENICOL DEGRADATION.
85.36° C.
pH - 6.00 PHOSPHATE BUFFERS
1/HALF LIFE - HOURS⁻¹
BUFFER CONCENTRATION - M/L

Fig. 6.—A plot showing the catalytic activity of monohydrogenphosphate ion upon the rate of hydrolytic degradation of chloramphenicol in *p*H 6.00 buffered aqueous solutions mantained at 85.36° C.

over, that when the antibiotic is exposed to solutions of strong acids, hydrolytic cleavage readily occurs with the formation of the corresponding amine (3).

As part of the present investigation, the catalytic activity of hydrogen ions was determined at relatively high concentrations. Perchloric acid solutions, ranging in concentration from 0.025–0.2 *M*, were employed as the source of the ion. This acid was chosen because of its very low volatility and because it is virtually totally dissociated in these concentrations.

The results of degradation effected in these media are summarized in Figs. 3–5. The broken circles represent the magnitude of the uncatalyzed reaction —these values being obtained by extrapolation to zero buffer concentration at *p*H 6.00 and 4.65 as shown in Figs. 6–9. They conclusively demonstrate that specific hydrogen-ion catalysis plays a major role in the degradation of chloramphenicol at sufficiently low *p*H values.

It is of particular interest to note that extrapolations of the straight line portions of Figs. 3–5 pass through the origin. This can be interpreted only as resulting from a quenching action of hydrogen ion on the uncatalyzed reaction. This behavior can be rationalized on the basis that in the presence of sufficiently high concentrations of hydrogen ion there is a change in the nature of the amide substrate. The results of a study of the effect of hydrogen-ion concentration upon the apparent solubility of the antibiotic supports this line of thinking. Figure 10 shows an increase in the apparent solubility of the drug at 30° with increasing concentrations of hydrogen ion. This increase is most logically attributable to the basic character of the amide linkage resulting in the formation of a protonated intermediate.[2]

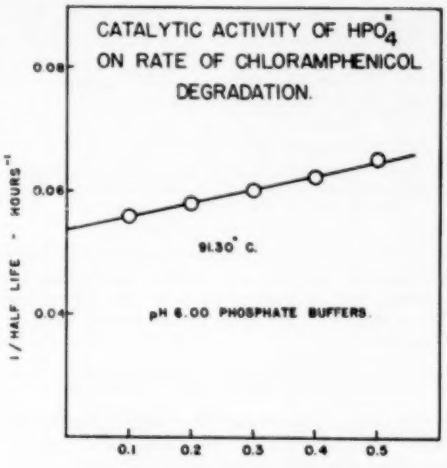


Fig. 8.—A plot showing the catalytic activity of monohydrogenphosphate ion upon the rate of hydrolytic degradation of chloramphenicol in *p*H 6.00 buffered aqueous solutions at 91.30° C.

Postulation of a mechanism to account for the hydrogen-ion catalyzed hydrolysis of chloramphenicol is rather difficult. A scheme such as the follow-

[2] Although the solubility study was carried out at 30°C and the kinetic studies at much higher temperatures, the qualitative picture is probably the same.

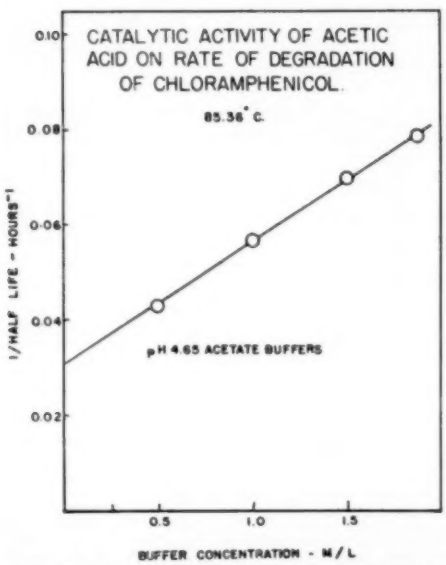


Fig. 7.—A plot showing the catalytic activity of undissociated acetic acid on the rate of hydrolytic degradation of chloramphenicol in *p*H 4.65 buffered aqueous solutions maintained at 85.36° C.

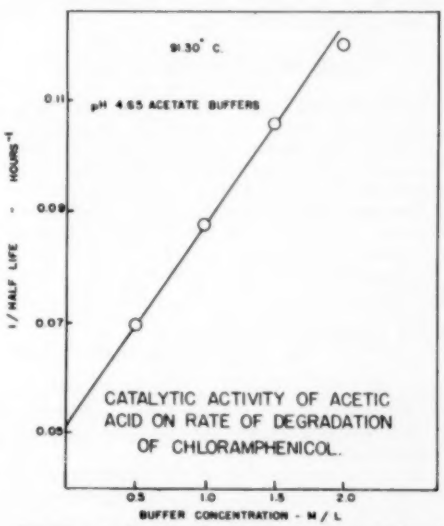
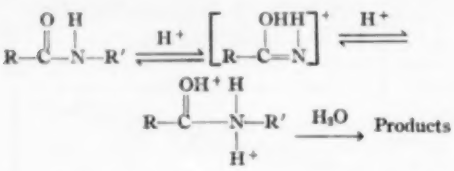


Fig. 9.—A plot showing the catalytic activity of undissociated acetic acid upon the rate of hydrolytic degradation of chloramphenicol in *p*H 4.65 buffered aqueous solutions at 91.30° C.

ing would appear, however, to represent the course of the reaction:



Despite the possible apparent theoretical difficulty involved in the formation of the doubly charged complex, this probably represents the mechanism of specific hydrogen-ion catalyzed hydrolysis most accurately.

TEMPERATURE DEPENDENCY

The Uncatalyzed (Water) Reaction.—From previously reported data (1) and from the values of reciprocal half lives obtained by extrapolation to zero buffer concentration in Figs. 6–9, it is possible to calculate the temperature dependency of the uncatalyzed hydrolytic cleavage reaction. Figure 11 shows that the reaction at both *p*H 6.00 and 4.65 yields a typical Arrhenius type plot. The heats of activation as determined from the slopes of these lines were 24.0 and 24.4 Kcal./mole. This slight variation is well within the limits of the experimental techniques.

The Hydrogen-Ion Catalyzed Reaction.—The temperature dependency of the hydrogen-ion catalyzed reaction, expressed as a second order reaction, is shown in Fig. 12. The catalytic constants[1] expressed as reciprocal half lives, with the dimensions liters moles^{-1} hours^{-1}, were determined from the slopes of the straight line portions of Figs. 3, 4, and 5. The heat of activation for this reaction as determined from the slope of the Arrhenius plot in Fig. 12 was 19.5 Kcal./mole.

GENERAL RATE EQUATION

As determined by the present and previous investigations (1, 2), the degradation of chloramphenicol in aqueous solution is represented by no single reaction. Even in systems in which the rate of degradation is uncomplicated by hydrolytic cleavage of the carbon-chlorine bonds (2), the observed rate constant represents the sum of a number of catalytic constants. For aqueous solutions below *p*H 7, the observed rate of loss of activity, k_{obs}, may be given as

$$k_{obs} = k_{H_2O}[H_2O] + k_{H^+}[H^+] + k_{OH^-}[OH^-] + [k_{HB}[HB] + [k_B[B]$$

where k_{H_2O}, H_{H^+}, k_{OH^-}, k_{HB}, and k_B are constants for the uncatalyzed, hydrogen-ion, hydroxyl-ion, general-acid and general-base catalyzed reactions, respectively.

Since the contribution of any catalytic species depends upon the *p*H of the system as well as the concentration and nature of the catalyst, the observed rate constant may often be represented more simply. In the case of those degradations carried out in the presence of moderate concentrations of a totally dissociated acid, for example, the above equation may be written

$$k_{obs} = k_{H^+}[H^+]$$

[1] It is not entirely accurate to use the term "catalytic constant." The values used in calculating the slopes of the plots in Figs. 3, 4, and 5 were actually reciprocal half lives. These half-life values are, however, directly proportional to the values of the corresponding rate constants and the slopes of the lines remain unchanged.

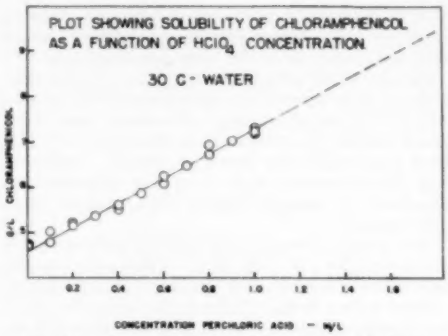

PLOT SHOWING SOLUBILITY OF CHLORAMPHENICOL
AS A FUNCTION OF HClO4 CONCENTRATION
30 C° WATER
G/L CHLORAMPHENICOL
CONCENTRATION PERCHLORIC ACID — N/L

Fig. 10.—A plot showing the effect of hydrogen ion concentration upon the apparent solubility of chloramphenicol in water at 30° C. It may be assumed that at this temperature and in these concentrations the perchloric acid used as a source of hydrogen ions is completely dissociated.

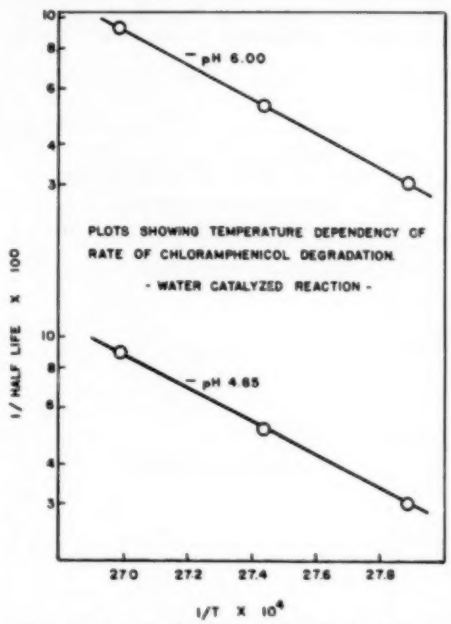


Fig. 11.—Arrhenius-type plots illustrating the temperature dependency of the uncatalyzed (water) reaction responsible for hydrolytic cleavage of chloramphenicol at its amide linkage in aqueous solutions at *p*H 6.00 and 4.65. The points shown were obtained by extrapolation to zero buffer concentration at the respective *p*H values.

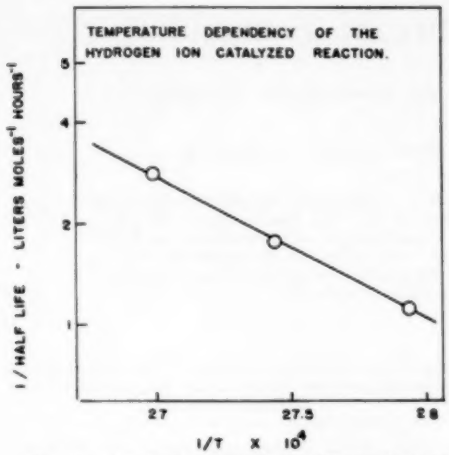


Fig. 12.—An Arrhenius-type plot illustrating the temperature dependency of hydrogen ion catalyzed hydrolysis of the amide linkage in chloramphenicol. The reaction is expressed as being of the second order.

TABLE I.—RATE CONSTANTS FOR THE UNCATALYZED (WATER) REACTION[a]

Temperature, °C.	k, Hours^{-1}
97.30	0.0631
97.30	0.0651
91.30	0.0371
91.30	0.0371
85.36	0.0212
85.36	0.0212

[a] Two values for the rate constant are presented at each temperature, because the rate constant of the uncatalyzed reaction was determined by extrapolation to zero buffer concentration at both pH 6.00 and 4.65.

TABLE II.—CATALYTIC CONSTANTS FOR THE HYDROGEN-ION CATALYZED REACTION

Temperature	k_H, Liters Moles^{-1} Hours^{-1}
97.30	2.00
91.30	1.223
85.59	0.792

The rate constants of the uncatalyzed reaction and the catalytic constants for the hydrogen-ion catalyzed reaction have been evaluated and are presented in Tables I and II. Table III gives the approximate values for the catalytic constant of undissociated acetic acid in the hydrolytic reaction. Although experimental data have been obtained for the catalytic activity of the general base HPO= magnitude of its catalytic effect was too small to permit evaluation or reasonable approximation. The catalytic activities of any other general acids or bases must be determined separately by specific experimentation.

TABLE III.—CATALYTIC CONSTANTS FOR ACETIC ACID CATALYZED REACTION[a]

Temperature	k_{HOAc}, Liters Moles^{-1} Hours^{-1}
97.30	0.058
91.30	0.044
85.36	0.034

[a] Since these values were obtained from systems varying in buffer capacity but adjusted to the same pH values, they represent only a good approximation.

GENERAL DISCUSSION

As is apparent from these studies the degradation of chloramphenicol in aqueous solutions is obviously a complicated phenomenon representing a summation of many individual reactions. Separation and study of the several degradative pathways have, however, been achieved. Our studies have so far been confined to degradative mechanisms operating in neutral and acidic systems. Other studies are presently in progress concerning degradation in alkaline media.

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Studies on the Stability of Fumagillin*

II. Photolytic Degradation of Crystalline Fumagillin

By THOMAS E. EBLE and EDWARD R. GARRETT

The tetraenedioic chromophore of fumagillin is destroyed by photolysis of the crystalline form in the presence and absence of air. An initial change in ultraviolet spectra occurs in both cases and the chromophore as measured at all wavelengths is then destroyed by a first order rate. Photolysis in the presence of air is accompanied by oxidation. Photolysis in the absence of air produces a material very readily oxidized in contact with air.

IT HAS BEEN SHOWN that fumagillin in ethanol solution is readily destroyed by light below 400 μ(1). Preliminary studies had also shown that photolytic degradation was not unique for the material in solution. Crystalline fumagillin exposed to light and air for one year lost 90 per cent of its absorptivity at 351 mμ(2). This paper concerns the kinetics of degradation of crystalline fumagillin in the presence and absence of air.

EXPERIMENTAL

Photolytic Degradation of Crystalline Fumagillin in the Presence of Air.—Two grams of fumagillin was ground in a mortar to a fine powder; 40-mg. samples were placed in the recesses of well slides.[1] A cover glass was secured over each sample with cellophane tape. One end of the tissue slide was solidly imbedded and glued to a rubb r stopper at a 40° angle to the vertical. The slides were then placed in the rack of the Atlas Fade-ometer[2,3] with the arc next to the smooth side and closest to the stopper.

Samples were removed at intervals and stored in brown glass bottles under nitrogen until analyzed spectrophotometrically[3] and chemically. The results of chemical analysis[4] of representative samples are given in Table I.

The carbon and hydrogen analyses parallel expected oxidation based on a fumagillin molecular weight of 472 (3).

Photolytic Degradation of Crystalline Fumagillin in the Absence of Air.—Two and one-half grams of fumagillin was dissolved in 50 ml. of chloroform. One-ml. aliquots (50 mg.) were pipetted into 5-ml. ampuls. The chloroform was evaporated to an oily residue by blowing nitrogen into each ampul while on a warm water bath. The oil was spread on one side of the ampul to a film and dried to a crystalline residue overnight in a vacuum desiccator. Oxygen free, dried nitrogen was bled into the desiccator. The ampuls were fire sealed immediately after nitrogen had been blown into each ampul and were then turned over and the sealed portion imbedded in a rubber stopper. The side with the film of dried fumagillin was adjacent to the carbon arc of the Atlas Fade-ometer and at a 40° angle to the vertical. The upper end of the ampul[5] was directed toward the arc.

Samples were removed at intervals and analyzed spectrophotometrically and chemically. The results of chemical analyses of representative samples are given in Table II.

A noticeable darkening of the material of Table II was observed on exposure to air after breaking the ampul. The acetyl analyses of the last three samples in the table were run after these materials had been exposed to air for some time (5).

TABLE I.—CHEMICAL ANALYSES OF CRYSTALLINE FUMAGILLIN[a] PHOTOLYTICALLY DEGRADED IN THE PRESENCE OF AIR

Hours of Exposure	Carbon, %	Hydrogen, %	Acetyl, %	Fumagillin by U. V. Assay at 351 mμ, %
Original	...	...	...	100 (assigned)[b]
1	64.26	7.00	1.99	98.6
5	62.51	6.93	2.74	68.7
12	60.51	6.79	3.08	50.0
30	60.31	6.88	3.87	41.0
52	59.67	6.75	5.11	39.5

[a] Fumagillin had been analyzed as 67.88% C, 7.45% H, and 0.0% acetyl (3).

[b] This material had $a_{351\ m\mu}$ = 110 (4) in 95% ethanol at zero time. Pure fumagillin is now assigned an absorptivity (a) of 147.5 at 351 mμ.

RESULTS AND DISCUSSION

Rate Studies on Photolytic Degradation of Crystalline Fumagillin.—The nature of the rate curves for photolytic degradation of crystalline fumagillin in the presence and in the absence of air are similar. Minor variations can be attributed to differences in techniques and transmissions of the glass containers. Table III records the asymptotic values of apparent absorptivities at the same wavelengths for the two studies.

Figure 1 is a plot of the logarithm of the apparent absorptivity (a in L./Gm.-cm.) against time in

* Received April 5, 1954, from the Research Division, The Upjohn Company, Kalamazoo, Mich.

[1] The glass slide had absorbances against air from 370 mμ through 270 mμ, at 10 mμ intervals of: 0.240, 0.240, 0.245, 0.255, 0.285, 0.345, 0.470, 0.730, 1.20, 1.91, and 2.85.

[2] The authors are indebted to F. H. Bither and J. R. Kramer for the use and operation of the Fade-ometer.

[3] The methods of spectrophotometric measurement and the use of the Atlas Fade-ometer have already been reported (1).

[4] The authors are indebted to W. A. Struck and associates of our analytical laboratory and to Howard S. Clark of Urbana, Ill., for the chemical analyses.

[5] Each wall of an ampul had absorbances against air from 370 mμ through 280 mμ, at 10 mμ intervals of: 0.240, 0.240, 0.250, 0.265, 0.297, 0.360, 0.490, 0.770, 1.35, and 2.50.

TABLE II.—CHEMICAL ANALYSES OF CRYSTALLINE FUMAGILLIN[a] PHOTOLYTICALLY DEGRADED IN ABSENCE OF AIR

Hours of Exposure	Carbon, %	Hydrogen, %	Acetyl, %	Fumagillin by U. V. Assay at 351 mμ, %
0.0	66.00	7.30	...	100 (assigned)[b]
3	67.19	7.41	1.84	81.6
6	66.37	7.41	1.65	67.0
9	66.97	7.45	1.34	43.0
12	66.65	7.51	1.08	39.1
15	66.85	7.33	1.23	36.6
20	66.83	7.76	2.98	36.5
30	66.55	7.42	3.71	32.6
50	66.11	7.48	5.40	31.0

[a] Fumagillin had been analyzed for 67.88% C, 7.45% H, and 0.0% acetyl (3).

[b] This material had $a_{351\ m\mu}$ = 123 in 95% ethanol at zero time.

hours of photolytic degradation of crystalline fumagillin in the presence of air. The results of the study under nitrogen were similar. The similarity of the curves at all wavelengths, except before five hours, is apparent.

Absorbances at wavelengths above 300 mμ decrease with time. Absorbances at all wavelengths below 300 mμ initially increase with time to maximum values. These values, however, are reached in one-third the time of solution photolysis (1). Maximum values for the absorbances at these lower wavelengths appear before the 351 mμ absorbance has become constant (Fig. 1) or before the $A_{(t-\infty)}$ absorbance difference has disappeared (Fig. 2). This differs from solution photolysis where the absorbance at the 351 mμ peak has completely disappeared when the absorbances at the lower wavelengths are at their greatest.

The initial absorptivity at 351 mμ of the crystalline fumagillin samples as prepared may indicate the presence of a diluent that manifests or affects anomalous initial changes in absorbance on photolysis. However, it is more probable that these observed changes represent a minor modification (F') in part or all of the fumagillin (F) in the crystalline lattice. The gross aspects of the fumagillin spectra are preserved, i. e., a diminution, but not elimination, of the 336 and 351 mμ maximum occurs. There is a slight increase in absorbance below 300 mμ, but without the appearance of any new discrete maxima. The logarithmic plots of the figures, of course, exaggerate the extent of this absorption increase.

TABLE III.—FINAL ASYMPTOTIC VALUES OF APPARENT ABSORPTIVITIES OF CRYSTALLINE FUMAGILLIN PHOTOLYTICALLY DEGRADED IN PRESENCE AND ABSENCE OF AIR

mμ	Under Nitrogen	Under Air
360	24	28
351	39	44
336	45	48
320	39	34
300	26	17
280	17	9
260	22	9
240	18	10

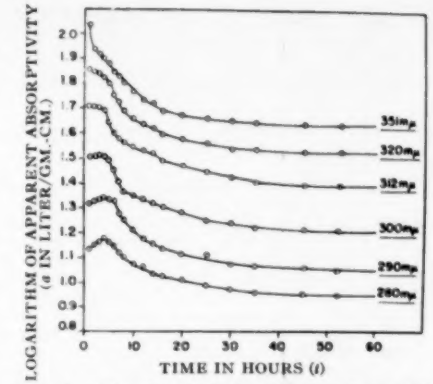

Fig. 1.—The photolytic degradation of crystalline fumagillin. Plot of the logarithm of the apparent absorptivity (a in liter/Gm.-cm.) at various wavelengths against time (t) in hours.

If the amount (dF') of modified fumagillin reacted within a time (dt) is proportional to the amount of modified fumagillin, $F' = F_0'$ Gm. at $t = 0$, then[6]

$$-dF'/dt = kF' \qquad (1)$$

The total absorbance (A_t) at any time (t) for each sample dissolved in (L) liters of 95% ethanol is:

$$A_t = [a_1(F' + F_\infty) + a_2(F_0' - F')]/L \qquad (2)$$

where a_1 and a_2 are absorptivities for fumagillin and its degradation product, respectively, and F_∞ is the amount of fumagillin in the sample never subjected to light. The absorbance (A_∞) of the estimated asymptotic value is the absorbance after all possible photolytic degradation has occurred which may be:

$$A_\infty = [a_1F_\infty + a_2F_0']/L \qquad (3)$$

The difference in absorbances ($A_{(t-\infty)}$) is:

[6] A possible explanation of observed first order kinetics of photolytic degradation of modified fumagillin (F') may be based on the mechanism

$$F' + h\gamma \underset{k_3}{\overset{k_1}{\rightleftarrows}} F'^* + F' \text{ (or } F) \xrightarrow{k_2} P \qquad (1)$$

where P is the product and the asterisk (*) represents the photolytically activated state. The number of fumagillin molecules in the light path is more than equivalent to the numbers of molecules in several cm. of a 4 mg./ml. solution of fumagillin which absorbs 99% of incident effective light within 50 μ of depth (1). Thus the steady state condition, $dF'^*/dt = 0$, would be met by

$$k_1I_{abs} = (k_2 + k_3F')F'^* \qquad (2)$$

where I_{abs} is the intensity of light absorbed and F' and F'^* are the densities in space. The rate determining step may be one wherein the activated molecule reacts with, or transfers its energy to, another molecule in the crystalline lattice. Since deactivation is more probable with lessened numbers of unreacted molecules adjacent,

$$-dF'/dt = dP/dt = k_2F'F'^* \qquad (3)$$

Substitution of the value of F^* from the Eq. 2 into Eq. 3 gives

$$-dF'/dt = k_1I_{abs}F'/(F' + k_3/k_2) \qquad (4)$$

If this mechanism is valid, first order kinetics result when $k_2 >> k_3$, i. e., when the rate of deactivation greatly exceeds the rate of product (P) formation and

$$-dF'/dt = kF' \qquad (5)$$

and the overall kinetics are first order with respect to the modified fumagillin.

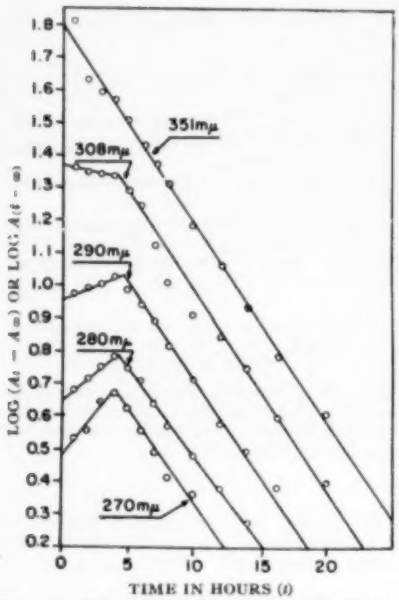

Fig. 2.—The pseudo first order photolytic degradation of crystalline fumagillin. Plot of the logarithm of the difference between absorbance (A_t) at time (t) and the asymptotic absorbance (A_∞) i. e., log $A_t - A_\infty$ or log $A_{(t-\infty)}$ against time (t) in hours.

$$A_{(t-\infty)} = A_t - A_\infty = (a_1 - a_2)F'/L \qquad (4)$$

Equation 4 may be substituted into Eq. 1 and the result simplified:

$$d(A_{(t-\infty)})/dt = -kA_{(t-\infty)} \qquad (5)$$

This equation integrates to

$$\log A_{(t-\infty)} = -kt/2.303 + \log A_{(t_0-\infty)} \qquad (6)$$

Thermal oxidation or photo oxidation should not significantly affect the kinetics of chromophore change since the same phenomena occurred under nitrogen.[7] If Eq. 6 is valid, the slopes of straight lines made by log $A_{(t-\infty)}$ *vs.* time in Fig. 2 are measures of the first order degradation rates of the modified fumagillin (F') present after five hours. The absorbances for all wavelengths disappeared at the same first order rates (k = 0.14 hr.$^{-1}$) for the 0.061 slopes of the straight lines in Fig. 2 where k is defined by Eq. 6. Peculiarly, this is very similar in magnitude to the first order rate constant for fumagillin degradation (to "neofumagillin") in solution when light of the same intensity is totally absorbed (1). This may indicate the same intermediate which is readily transformed by solvolytic action in solution to several chromophores ("neofumagillins") but cannot be so in the crystalline lattice.

Photolyzed Fumagillin in Air and Nitrogen.—The major difference between fumagillin photolyzed in the presence and absence of air appears to be in the chemical analyses of the degradation products. In both cases the fumagillin chromophore is destroyed at about the same first order rate.

The formation of products which are susceptible to oxidation and are independent of the chromophore must occur. In the presence of air these products react with oxygen as they are formed. Under nitrogen they are also formed and react on exposure to air.

SUMMARY

1. Crystalline fumagillin is readily destroyed by photolysis. There is no great difference in the mode of tetraenedioic chromophore destruction in the presence and absence of air.

2. Absorbances at wavelengths below 300 mμ increase as the fumagillin chromophore is destroyed. No new chromophore can be recognized. These increased absorbances are also destroyed at the same rate as the absorbances at the characteristic maxima of fumagillin.

3. Photolysis of crystalline fumagillin in the presence of air is accompanied by oxidation and increase in acetyl content, most probably in the alcohol portion of the molecule.

4. Photolysis of crystalline fumagillin in the absence of air produced a material very readily oxidized when subsequently brought in contact with air.

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(2) Eble, T. E., unpublished data.
(3) Eble, T. E., and Hanson, F. R., *Antibiotics and Chemotherapy*, **1**, 54(1951).
(4) All spectrophotometric terms, definitions, and symbols used in this paper follow that of "Suggested Nomenclature in Applied Spectroscopy," Hughes, H. K., *Anal. Chem.*, **24**, 1349(1952).
(5) Howard S. Clark, Urbana, Illinois, private communication.
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[7] Professor T. Higuchi, University of Wisconsin, has suggested that it should be stressed that the mechanism is probably closely related to photochemical "drying" (polymerization) of polynoid oils. Such light-catalyzed polymerization takes place at the double bonds and is free radical in nature (6). Air drying may be a simultaneous reaction, but of less relative significance in this study. These mechanisms are not excluded by the discussion in Footnote 6.

Studies on the Stability of Fumagillin*

III. Thermal Degradation in the Presence and Absence of Air

By EDWARD R. GARRETT

The tetraenedioic chromophore of crystalline fumagillin is thermally destroyed in the presence and absence of air by different mechanisms. The degradation in air is characterized by rates that are second order with respect to fumagillin and can be explained by the reaction of one molecule of oxygen with two molecules of fumagillin. This reaction may completely transform all the fumagillin. The degradation under nitrogen is initially pseudo zero order at all temperatures to about 80 per cent of the absorptivity of pure fumagillin. Further chromophore degradation is not linear with time and the extent is a function of temperature. The rate constants have been evaluated for both types of chromophore degradation at 70, 60, 50, and 40° and the heats of activation have been calculated. The fumagillin chromophore in ethanol solution under nitrogen undergoes slight initial modification and is more stable than in the crystalline form.

IT HAS BEEN SHOWN that fumagillin is degraded by photolysis in solution and crystal in the presence or absence of air (1, 2). Photolysis was more responsible than photo-oxidation or simultaneous oxidation. In order to complete the stability picture on fumagillin, thermal effects in the absence of light in both the crystalline and soluble states were studied in the presence and absence of air.

EXPERIMENTAL[1]

Thermal Degradation of Crystalline Fumagillin.—One milliliter aliquots of a solution of fumagillin, 5 Gm./100 ml. $CHCl_3$, were pipetted into 5-ml. ampuls. The solvent was removed under vacuum. Half the ampuls were left open to the air and half were purged three times with nitrogen and subsequently sealed. Both sets of ampuls were distributed among 50, 60, and 70° (±0.5°) mineral oil constant temperature baths and a 40° (±1.0°) water constant temperature bath. Samples were removed at various times and absorbances (3) measured at various wavelengths in 0.01 mg./ml. solutions of 95% ethanol and apparent absorptivities calculated (i. e., a in liter/gram-cm.).

Thermal Degradation of Fumagillin in Ethanol Solution.—Aliquots of a 2 mg./ml. solution of fumagillin in 95% ethanol were sealed under nitrogen in ampuls. The experimental procedure was the same as for the crystalline fumagillin.

CALCULATIONS AND THEORY

Thermal Degradation of Crystalline Fumagillin in Presence of Air.—Logarithmic or nonlogarithmic plots of apparent absorptivities (a) of thermally degraded crystalline fumagillin in presence of air against time for a given temperature and wavelength were not linear. The kinetics are not zero or first order.

The best linear relation of a function of apparent absorptivities with time results when the reciprocal of the apparent absorptivity ($1/a$) is plotted against time. Figure 1 for 351 mμ is typical of the many such plots prepared for various wavelengths.

This pseudo bimolecular degradation may be explained by postulating an oxidation sequence such as:

$$F + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} F{\cdot}O_2 \qquad (1)$$

$$F{\cdot}O_2 + F \xrightarrow{k_2} 2FO \qquad (2)$$

where F is fumagillin, k_1 is the rate constant for formation of the activated oxygenated fumagillin, k_{-1} is the reverse rate of the equilibrium and k_2 is the rate constant for the irreversible formation of fumagillin oxide (FO).

The rate of appearance of the activated oxygenated fumagillin molecule (term a in Eq. 3) will depend on its formation and will be proportional to the concentration of oxygen (i. e., partial pressure)

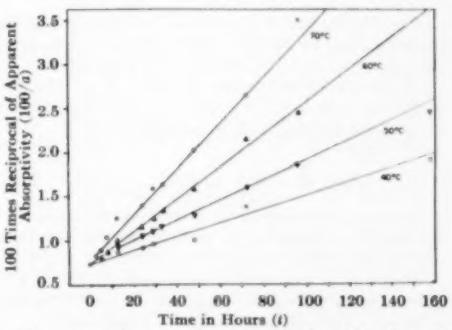

Fig. 1.—The pseudo bimolecular kinetics of crystalline fumagillin thermal degradation in presence of air. Plot of 100 times the reciprocal of the apparent absorptivity ($100/a$ with a in liter/Gm.-cm.) at 351 mμ against time in hours for several temperatures.

* Received April 5, 1954, from the Research Division, The Upjohn Company, Kalamazoo, Mich.

[1] The cooperation of Thomas E. Eble of the Research Division, The Upjohn Company, is gratefully acknowledged.

and the concentration in space (i. e., in the crystal lattice) of fumagillin (term b in Eq. 3). This rate of appearance will be slowed, however, by the reversible breakup rate of the oxygenated fumagillin (term c) and the consumption in fumagillin oxide formation (term d).

$$\underset{(a)}{d[F{\cdot}O_2]/dt} = \underset{(b)}{k_1[F][O_2]} - \underset{(c)}{k_{-1}[F{\cdot}O_2]} - \underset{(d)}{k_2[F{\cdot}O_2]F} \quad (2)$$

The rate of disappearance of fumagillin (term a in Eq. 4) will depend on its consumption of oxygen (term b) and reaction with activated oxygenated fumagillin (term d). This rate will be slowed by the reversible breakup of the oxygenated fumagillin (term c).

$$\underset{(a)}{-d[F]/dt} = \underset{(b)}{k_1[F][O_2]} - \underset{(c)}{k_{-1}[F{\cdot}O_2]} + \underset{(d)}{k_2[F{\cdot}O_2][F]} \quad (4)$$

A generally accepted solution (4) of these equations is to introduce the steady state approximation:

$$d[F{\cdot}O_2]/dt = 0 \quad (5)$$

This is based on the premises that $[F{\cdot}O_2]$ is small and the rate of its change as the reaction proceeds will be small. Rates of formation are equal to rates of decomposition when Eq. 5 is substituted into Eq. 3. The resultant solution for $[F{\cdot}O_2]$ is:

$$[F{\cdot}O_2] = k_1[F][O_2]/(k_{-1} + k_2[F]) \quad (6)$$

This may be substituted into Eq. 4 so that

$$-d[F]/dt = 2k_1k_2[F]^2[O_2]/(k_{-1} + k_2[F]) \quad (7)$$

If the oxygen concentration is constant then $[O_2] = k_3$ and

$$-d[F]/dt = 2k_3k_1k_2[F]^2/(k_{-1} + k_2[F]) \quad (8)$$

If $k_2[F] >>> k_{-1}$, that is, if the rate of decomposition of the activated oxygenated fumagillin $[F{\cdot}O_2]$ is so much slower than the rate of fumagillin oxide formation that k_{-1} can be disregarded,

$$-d[F]/dt = 2k_1k_3[F] \quad (9)$$

The plot of log of absorbance *vs.* time should be linear of slope $(-2k_1k_3)$. This is not so; the kinetics were not first order with respect to fumagillin.

If the rate determining step were Eq. 2 and the activated oxygenated fumagillin showed high rates of decomposition or low stability (i.e. $k_{-1} >>> k_2[F]$) then

$$-d[F]/dt = 2k_1k_2k_3[F]^2/k_{-1} = k[F]^2 \quad (10)$$

These kinetics are second order with respect to fumagillin. These latter postulates give the best fit with the experimental data.

Equation 10 states that the rate of loss of fumagillin is proportional to the square of the fumagillin content. This equation may be integrated:

$$1/[F] = kt + 1/[F]_0 \quad (11)$$

Let a be the apparent absorptivity in liter/Gm.-cm. at any time (t) during thermal air degradation where a_0 is the absorptivity for pure fumagillin at zero time. Then, if no contaminating absorption arises, the fumagillin content at any time may be expressed as a fraction of the original:

$$[F] = a/a_0 \text{ and } [F]_0 = 1 \quad (12)$$

Equation 11 becomes

$$1/a = kt/a_0 + 1/a_0 = k't + 1/a_0 \quad (13)$$

The plot of the reciprocal of the apparent absorptivity against time should be linear of constant slope k' where k' is a function of the wavelength of the absorptivity of pure fumagillin at that wavelength.

The rate constant, k' and the derived k of Eq. 13, were obtained from linear plots as in Fig. 1 and in other similar figures drawn for other wavelengths. It was noted, that for such plots as 336 mμ and below, an initial deviation from the pseudo second-order relation exists within the first ten hours. This deviation is a greater than expected initial decrease in absorptivity and worsens with decreasing wavelength. The reciprocal plot of data obtained at greater than ten hours does conform to linearity, however.

TABLE I.—TABLE OF RATE CONSTANTS (k' AND $k^a = a_0k'$) AS DETERMINED FROM PLOTS OF RECIPROCAL OF APPARENT ABSORPTIVITIES ($1/a$) *vs.* TIME FOR THERMAL DEGRADATION OF CRYSTALLINE FUMAGILLIN IN PRESENCE OF AIR

Temperature, ° C.	Wavelength, mμ	$10^4\,k'$	$10^2\,k$
70	360	4.2	3.8
	351	2.6	3.5
	336	2.2	3.2
	320	2.6	2.5
	305	2.8	1.4
60	360	2.8	2.5
	351	1.9	2.6
	336	1.7	2.5
	320	2.1	2.0
	305	2.0	1.0
50	360	1.7	1.5
	351	1.2	1.6
	336	1.0	1.5
	320	1.3	1.3
	305	1.3	0.6
40	360	0.9	0.8
	351	0.7	1.0
	336	0.4	0.6
	320	0.9	0.8
	305	0.8	0.4

[a] a_0 was 90.3, 136.6, 148.6, 98.5, and 49.0 at 360, 351, 336, 320, and 305 mμ, respectively.

The constancy of the rate constant, k, is apparent at 360, 351, and 336 mμ. The experimental error was greatest at 40°. These samples were exposed to water-saturated air. The decrease in k with lower wavelengths may be explained by postulating a degradation product (x) that contributes to absorbance below 336 mμ. This degradation product may arise at the same rate that the fumagillin chromophore disappears and its rate expression would be similar to Eq. 11.

$$1/([F]_0 - x) = kt + 1/[F]_0 \quad (14)$$

The observed or apparent absorptivity (a) would then be the sum of the absorbance contributions of the residual fumagillin $[F]$ and the product (x).

Equations 11 and 14 may be used and a_x is the absorptivity of the product (x).

$$a = a_0[F] + a_x x = [F]_0(a_0 + a_x[F]_0 kt)/(1 + [F]_0 kt) \quad (15)$$

$[F]_0$ may be considered as unity to determine the relation between the fraction of the total or apparent absorptivity (a) contributed by the fumagillin and the fractions contributed by the products at any time (t).

$$a_0/a = 1 - (a_x/a - 1)(kt) \quad (16)$$

The a_0/a values may be plotted against kt to provide a family of curves for various values of the ratio (a_x/a_0) of the absorptivity of the degradation product (x) to the absorptivity of fumagillin (F).

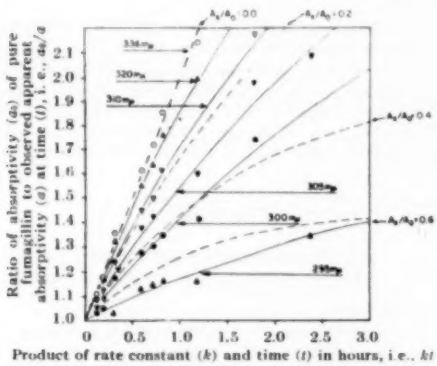
Ratio of absorptivity (a_0) of pure fumagillin to observed apparent absorptivity (a) at time (t), i. e., a_0/a
Product of rate constant (k) and time (t) in hours, i.e., kt
a_x/a_0=.00
a_x/a_0=.02
a_x/a_0=.04
a_x/a_0=.06
336 mμ
320 mμ
305 mμ
300 mμ
290 mμ

Fig. 2.—Plot of the ratio (a_0/a) of the absorptivity (a_0) of pure fumagillin to the observed or apparent absorptivity (a) at various wavelengths against the product (kt) of the bimolecular rate constant (k = 0.025) at 60° C. and time (t) in hours of the observation. The dashed curves are the theoretical relations for labeled various ratios (a_x/a_0) of the absorptivity (a_x) of the oxygenated fumagillin degradation product to the absorptivity (a_0) of pure fumagillin.

The labeled curves of Fig. 2 are the theoretical plots against kt for a_x/a_0 = 0.0, 0.2, 0.4, and 0.6, i. e., the wavelengths where the resultant degradation product has no absorbance, 1/5 that of fumagillin, 2/5 etc. Figure 2 for the 60° data is typical of the plots made for the data of all temperatures. The data at 336 mμ confirm the expectation that $a_x/a_0 = 0$ or that the degradation product has little absorptivity at this wavelength. The a_x/a_0 values at other wavelengths may be estimated from their formal resemblance and position between the dashed lines representing the theoretical curves for various values of a_x/a_0.

The isobestic point is at about 290 mμ where there is no significant change in absorbance with time. Below this wavelength there are slight increases in absorbance to indicate that the product has slightly higher absorptivities than fumagillin.

Table II lists the a_x/a_0 ratios so determined. A plot of a_x *vs.* wavelength would provide an estimate of the ultraviolet spectra of the air degradation product.

TABLE II.—ESTIMATED RATIOS (a_x/a_0) OF ABSORPTIVITIES OF FUMAGILLIN DEGRADATION PRODUCT TO FUMAGILLIN

Wavelength, mμ	Temperature, ° C. 70	60	50	a_0	a_x
336	0.00	0.00	0.00	148.6	0.0
320	0.05	0.05	0.05	98.5	4.5
310	0.17	0.17	0.20	59.6	10.7
305	0.25	0.25	0.30	49.0	12.7
300	0.40	0.40	0.40	36.3	14.5
295	0.60	0.63	0.65	25.0	15.8
290	1.00	1.00	1.00	19.0	19.0
285	1+	1+	1+	13.0	17.0

The Arrhenius plot of the logarithm of rate constant (k') against the reciprocal of the absolute temperature (T) is given in Fig. 3 for the rates measured at 351 mμ. The equation of best fit is:

$$\log k'_{351\ m\mu} = -1962/T + 2.14 \quad (17)$$

The standard deviation of a predicted log k' at a given temperature is 0.020 and the standard deviation of the slope is 92. The heat of activation is 9.0 Kg. cal./mole. The heats of activation for rates measured at 305, 320, and 336 mμ were not statistically significantly different than that at 351 mμ. However, the variances in log k' and due to regression were the least for 351 mμ. Again this demonstrates the least variability in using 351 mμ as the wavelength for studying changes in the fumagillin chromophore (1).

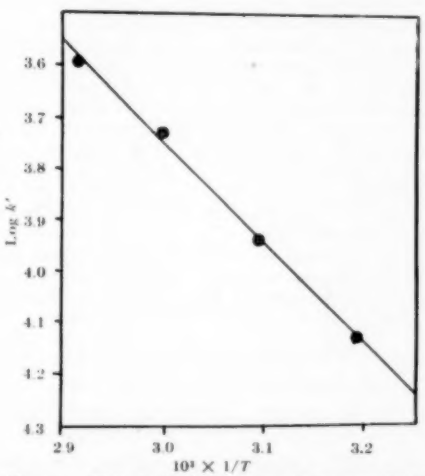

Fig. 3.—Plot of the logarithm of the pseudo bimolecular rate constant (k') for the thermal oxidation of fumagillin against the reciprocal of the absolute temperature (T) as obtained from spectral data at 351 mμ.

Thermal Degradation of Crystalline Fumagillin in Absence of Air.—The plot of apparent absorptivity against time for thermally degraded crystalline fumagillin under nitrogen is shown in Fig. 4. Plots at other wavelengths above 310 are similar

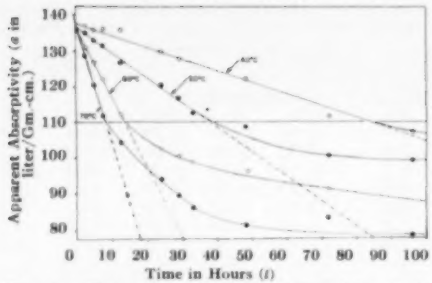

Fig. 4.—Plot of the apparent absorptivity (a in liter/Gm.-cm.) at 351 mμ against time in hours (t) for thermally degraded fumagillin in the absence of air.

to this one at 351 mμ but 295 mμ is an isobestic point. Below this wavelength absorptivities increase at fast initial rates and then more slowly. This again indicates the appearance of a new chromophore at the lower wavelengths.

The mechanism of thermal degradation under nitrogen is obviously complex. An initial pseudo zero order rate is as consistent with the data as is any other low order kinetics. Linear decrease of absorptivity in Fig. 4 occurs until a definite value (about $a = 1.10$ or 80% purity) is reached. This value is apparently independent of temperature within the ranges studied. Fumagillin refrigerated under nitrogen for a year also showed an initial decrease that did not go below this value (5).

A new mode of degradation appears at higher temperatures when this value is reached. A nonlinear degradation continues below this value so that the possible asymptote appears to be some direct function of the temperature of degradation. Absorbances measured at 168 and 360 hours for the several temperatures were the same as the values given in Fig. 4 for 96 hours. The respective rates (R) of apparent absorptivity decrease to $a = 110$ at 351 mμ are for 70°, $R = 3.28$; for 60°, $R = 1.95$; for 50°, $R = 0.698$ and for 40°, $R = 0.329$ absorptivity units/hr.

The Arrhenius plot of the logarithm of the rate (R) against the reciprocal of the absolute temperature is given in Fig. 5 for the rates measured at 351 mμ down to $a = 110$. The equation is:

$$\log R_{(351\ m\mu)} = -3760/T + 12.95 \qquad (18)$$

The heat of activation is 17.2 Kg. cal./mole.

RESULTS AND DISCUSSION

Thermal Degradation of Crystalline Fumagillin.—Crystalline fumagillin in the presence of air must undergo a thermally activated reaction with oxygen which decreases the conjugation of the fumagillin chromophore. The kinetics are second order with respect to fumagillin and may be explained by the loose addition of a molecule of oxygen to a molecule of fumagillin. Subsequent bridging of the oxygen to the chromophore of an adjacent fumagillin would be the rate-determining step.[3]

[3] Professor T. Higuchi, University of Wisconsin, has suggested that it should be stressed that the mechanism is probably closely related to air "drying" of polyenoid oils (6).

The thermal activation of crystalline fumagillin in the absence of air is pseudo zero order initially. This indicates that a thermal modification of the chromophore in the crystalline lattice may be at first independent of the position of the fumagillin in the lattice. A similar initial change in the chromophore also occurred in the photolysis of crystalline fumagillin (2). The decrease of rate of chromophore degradation below a specific apparent absorptivity value indicates a possible new mechanism of subsequent thermal degradation. The unusual effect of temperature on the asymptotic value approached suggested a thermal equilibrium. The fact that this does not appear to occur readily on thermal activation in solution indicates that decrease in unsaturation may result from bonding of adjacent molecules in the crystal lattice. The extent of this bonding could be a function of temperature.

Thermal Degradation of Fumagillin in Solution.—The fumagillin chromophore is relatively stable in ethanol solution under nitrogen. Some initial degradation did occur to 82% of the original value based on absorptivity at 351 mμ. However, at 40, 50, 60, and 70°, samples taken from the ninth to the thirty-fifth day showed no loss in absorptivity.

SUMMARY

1. Crystalline fumagillin is destroyed by heat in the presence and absence of air by different mechanisms.

2. The thermal destruction of the tetraenedioic chromophore of crystalline fumagillin in air most probably involves the reaction of one molecule of oxygen with two molecules of fumagillin. Complete transformation to several chromophores in the further ultraviolet occurs.

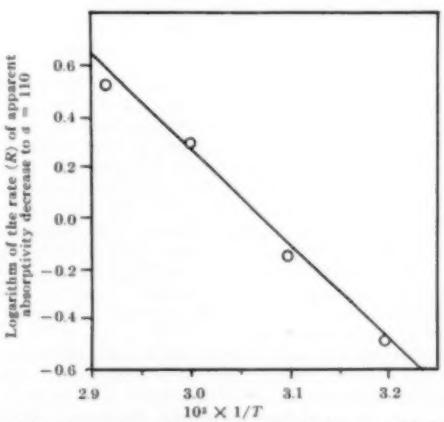

Fig. 5.—Plot of the logarithm of the rate (R in absorptivity units/hour) of decrease in fumagillin absorptivity (a) above $a = 110$ for thermal degradation of fumagillin in absence of air against the reciprocal of the absolute temperature (T) as obtained at 351 mμ.

3. The thermal destruction of the tetraenedioic chromophore of crystalline fumagillin in the absence of air is more complex. The initial degradation is pseudo zero order to about 80% of the absorptivity of pure fumagillin. Subsequent chromophore degradation is not linear with time and the extent is a function of the temperature.

4. After an initial thermal degradation, fumagillin in ethanol solution under nitrogen maintained 80% of its absorptivity even at 70° for the thirty-five days of study.

5. Procedures for the spectrophotometric study of polyene degradation have been developed.

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The *in Vitro* Evaluation of the Antibacterial Activity of Undi Oil (*Calophyllum inophyllum* Linn.)*

By S. G. BHAT, J. G. KANE, and A. SREENIVASAN†

Undi oil (*Calophyllum inophyllum* Linn.) has antibacterial activity *in vitro* against Gram-positive organisms. The active principle from the oil has been fractionated by alcohol extraction, molecular distillation, and chromatography and partially characterized.

SINCE ancient times some of the vegetable oils like Neem (*Azadirachta indica*), Undi (*Calophyllum inophyllum* Linn.), Karanja (*Pongamia glabra* Vent.), Malkanguni (*Celastrus paniculatus* Willd.) etc., have been used in certain human ailments especially against skin affections. In our studies on testing of these oils *in vitro* for antibacterial activity, Undi oil showed marked activity against Gram-positive organisms. The results together with observations on the separation of its active principle by alcohol extraction, molecular distillation, and chromatography are reported here. When this work was in progress, Ormancey-Potier, *et al.* (1), reported the isolation of certain products from the defatted kernels of *Calophyllum inophyllum* from Madagascar. They have found that the "Calophyllolide"—one of the isolated products—was active against *Mycobacterium tuberculosis*.

The oil from the seeds of *Calophyllum inophyllum* Linn. is dark green in color and possesses a disagreeable odor and taste. Because of the presence of nonfatty constituents it is unsuitable for edible purposes (2–4). Its high acidity is due partly to resin acids as reported by several investigators (2, 3, 5, 6–9). The oil is cheap and is mainly used for wood preservation (2,4) and for illumination purposes (2–5, 10–13). It has also been recommended in soap making (2,5,11,12). The oil has been reported to be useful in treatment of rheumatism (2,5,11–13), gleet (12), burns (5), gonorrhea (12), leprosy (2,14,15) and skin affections (2,10,14). The fatty acid composition of the oil has been investigated (8,9,16–18). The refined oil (13) or the ethyl esters of the fatty acids have been intramuscularly injected to relieve pains of leper patients. Georgi (14) reports that nothing is known regarding the active principle of this oil.

EXPERIMENTAL

Undi Oil.—The sample of oil obtained by crushing in ghanis (Bullock drive expeller) was secured from South Kanara, Madras State, India. The characteristics of the oil were as follows: Ref. index at 32°—1.4730; acid value—27.6; saponification value—197.1; iodine value (Wijs)—92.3; unsaponified matter (petroleum ether)—1.25%.

Test Organisms.—A twenty-four-hour culture of the test organism in 10 cc. of nutrient broth was used. The stock culture was subcultured on a fresh agar slant once a fortnight.

The sources of the organisms used were as follows: *Micrococcus pyogenes* var. *aureus* (209, FDA); *Bacillus subtilis; Micrococcus pyogenes* var. *albus* (isolated at the Microbiology Department, St. Xavier's College, Bombay); *E. coli* communior

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(National Collection Type Culture, India, 415); *Salmonella typhosa* (Ty. 2c. strain of Felix); *Salmonella paratyphi* (N. C. T. C., India, 5705); *Shigella dysenteriae* (shiga), *Streptococcus pyogenes*, *Streptococcus viridans*, *Diplococcus pneumoniae*, and *Corynebacterium diphtheriae* (isolated at the King Edward Memorial Hospital, Parel, Bombay).

Medium.—Nutrient broth of the following composition and of *p*H 7.4 was used throughout. Peptone 0.5%, meat extract (Lab-Lemco, Oxo Ltd., London) 0.3%, sodium chloride 0.5%, and glucose 1.0%; 2.5% agar was used for nutrient agar. Nutrient agar containing 5% sterile defibrinated blood was used for the exacting organisms.

Preparation of Oil Emulsion.—A known weight of the oil was emulsified in a hand homogenizer with a solution of 0.05% Tween® 80 (polyoxyethylenesorbitanmonooleate) containing 0.5% potassium carbonate which acts as an emulsion stabilizer (19) and solution made up to a known volume.

Test Methods for Antibacterial Action.—(*a*) The agar cup plate method of Heatley (20) as modified by Schmidt and Moyer (21) was followed with slight changes. The plates were seeded with 3–4% of an active twenty-four-hour culture of the test organism. Cups were bored with a sterile cork borer No. 4 (diameter of agar cup, 6.5 mm.); 0.1 cc. of the oil emulsion was carefully pipetted into the agar cups, along with a control cup containing the emulsifying solution without the sample of oil. The Petri plates were refrigerated for two hours and then incubated at 37° overnight. The diameter of zone of inhibition was measured and recorded; the control cups showed no zone of inhibition.

(*b*) The agar-streak assay described by Waksman and Reilly (22) was employed with some modifications.

A twenty-four-hour culture of the organism was streaked on set agar containing different concentrations of Undi oil. Three discrete streaks of each organism were made on agar without recharging the platinum loop which produced streaks approximately 1.5 to 2.0 cm. wide. The platinum loop was flamed, recharged between Petri plates and between organisms in each Petri plate. Control plates under similar conditions, with the emulsifying medium, were kept side by side. The Petri plates were incubated at 37° overnight and the results recorded. The growth of the bacterial culture on each plate was compared with their corresponding control plates.

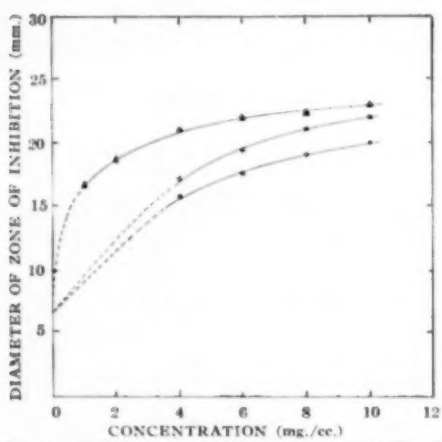

Fig. 1.—Activity curves of Undi oil. ▲—▲ *B. subtilis*; ×—× *M. albus*; ●—● *M. aureus*.

(*c*) The serial dilution method of Foster and Woodruff (23) was employed, with slight modifications.

The inoculum consisted of two drops of a twenty-four-hour culture diluted 50 times with sterile distilled water The tubes were incubated for twenty-four hours or longer. Since there was turbidity in the beginning when the test oil emulsion was added, the bactericidal concentration was determined by subculturing from each tube to fresh tubes containing 5 cc. of nutrient broth. These tubes were incubated at 37° overnight and the results were recorded. The dilution at which there was no growth on subculturing was taken to indicate the bactericidal concentration of the material tested.

The alcohol-soluble portions were dissolved in 95% ethyl alcohol instead of being emulsified. The control plates contained a similar amount of ethyl alcohol only.

FRACTIONATION OF UNDI OIL

Alcohol Extraction.—The oil (92 Gm.) was refluxed with ethyl alcohol (90%, v/v, at 30°) in the ratio of 1:4 on a water bath for four hours and the mixture was allowed to separate into two layers. The raffinate (lower layer) and the extract (upper layer) were freed from alcohol.

Molecular Distillation.—The oil (312 Gm.) was molecularly distilled using a cyclic falling film still (Hickman model type CMS 1000; supplied by Distillation Products, Inc., Rochester 13, N. Y.). Fractions were collected at different ranges of temperatures, and at each temperature range several cycles were collected in the same receiver.

Chromatography.—A preliminary experiment showed that the active principle could not be adsorbed on charcoal or Fuller's earth. However, it was found that the filtrate obtained by passing the oil in petroleum ether over alumina, showed no activity against *M. aureus* by the agar cup plate assay. Hence the chromatogram was developed over alumina with petroleum ether: 4.9 Gm. of molecularly distilled fraction between 130–181° of *Undi* oil in petroleum ether (b. p. 68°) was passed through a column (1-inch diameter x 12-inch length) filled with 47 Gm. of Brockmann's alumina (Grade IV). On addition of the fraction in petroleum ether the column turned yellow in color. The column was washed with petroleum ether till the filtrate was colorless and there was no separation of the bands.

Chloroform was passed through the column and a yellow band was found to come down. The addition of chloroform was continued till the filtrate was colorless.

Next, 95% ethyl alcohol was passed through and another yellow band was found to move down. This was collected separately, till the filtrate was colorless.

Ultraviolet Absorption.—This was determined in alcohol with a Beckman spectrophotometer (Model DU), using 1-cm. cell.

RESULTS AND DISCUSSION

Three samples of Undi oil obtained by crushing in ghanis, rendering, and solvent extraction, respectively, showed bacteriostatic activity (diameter of zone of inhibition, 21 mm., against *M. aureus* in a concentration of 10 mg./cc.) of the same order, which indicated that the method of extraction did not affect the activity of the oil.

The antibacterial activity of Undi oil was tested by the agar streak assay against some pathogenic and nonpathogenic organisms, of both Gram-positive and Gram-negative types. The oil was active against *M. aureus*, *B. subtilis*, *M. albus*, *S. pyogenes*, *S. viridans*, *D. pneumoniae*, and *C. diphtheriae*; and was not active against *E. coli*, *S. typhosa*, *S. paratyphi*, and *S. dysenteriae* (shiga). It showed antibacterial activity against seven Gram-positive organisms tested.

The agar cup plate assay of emulsified Undi oil at different concentrations was carried out and plots obtained for activity curves (see Fig. 1) against *M. aureus*, *B. subtilis*, and *M. albus*. The curves were extrapolated to the diameter of the agar cup, as the zones of inhibition were not clear beyond a certain concentration. Transfers made from the zones of inhibition into tubes containing nutrient broth showed no growth when incubated overnight, indicating that the action was also bactericidal.

The antibacterial activity of Undi oil by the agar cup plate, the agar streak, and the serial dilution assays are shown in Table I.

TABLE I.—ACTIVITY OF UNDI OIL BY DIFFERENT METHODS OF ASSAY

Assay	Active Concentration (mg./cc.) *M. aureus*	*B. subtilis*	*M. albus*
Agar cup plate	4.0	1.0	4.0
Agar streak	1.0	0.4	0.8
Serial dilution	3.0	2.0	3.0

The oil emulsion (10 mg./cc.) was stable for one month when stored at room temperature (27–29°), activity being the same as that of a fresh emulsion. Diameters of zones of inhibition were 21 mm., 25 mm., and 22 mm. against *M. aureus*, *B. subtilis*, and *M. albus*, respectively, the emulsifying solution giving no zone of inhibition.

The activity of Undi oil was also stable to autoclaving at a steam pressure of 15 lbs. per sq. inch for fifteen minutes.

The activities of alcohol-soluble (extract) and alcohol-insoluble (raffinate) portions were compared with that of the original oil. Undi oil showed a zone of inhibition of 18 mm. against *M. aureus* in a concentration of 7 mg./cc.; the extract showed the same diameter of zone of inhibition in a concentration of 0.5 mg./cc.; and the raffinate oil showed no zone of inhibition.

The alcohol extraction of Undi oil concentrates all the active principle in the extract and the activity is 14 times that of original oil against *M. aureus*. The raffinate oil had no activity though its acid value was 7.0. The unsaponifiable matter also had no antibacterial activity in a concentration of 10 mg./cc. The raffinate oil was split after saponification in alcohol, and the mixed fatty acids (10 mg./cc.) were tested against *M. aureus*. These had no activity which clearly indicates that the fatty acids of the oil had no activity and that the active principle is not a part of the glycerides. The adsorption of the active principle on alumina suggests that the material is acidic. The fact that it could be eluted with chloroform further shows that it is not a fatty acid but some acidic substance associated with the oil during its extraction from the seeds.

Molecular distillation of Undi oil was carried out to see the extent of separation of the active principle. The activities of the fractions collected were determined by the agar cup plate assay at a concentration of 10 mg./cc. The results are presented in Table II.

TABLE II.—ACTIVITIES OF MOLECULARLY DISTILLED FRACTIONS OF UNDI OIL (10 MG./CC.)

Fraction	Yield, %	Diameter of Zone of Inhibition in mm.[a] *M. aureus*	*B. subtilis*	*M. albus*
Below 130°	3.9	17.0	21.0	17.0
130–180°	11.5	23.0	32.0	25.0
180–220°	4.9	26.0	34.0	28.0
220–240°	11.0	18.0	21.0	17.0
240–250°	17.8	[b]	[b]	[b]
Residual oil	50.9	[b]	[b]	[b]
Original Undi oil	...	21.0	25.0	22.0
Control	[b]	[b]	[b]	[b]

[a] Average of at least six zones of inhibition.
[b] No zone of inhibition.

It is seen that maximum activity was shown by the fractions distilling between 130–180° and 180–220° against the test organisms. The last fraction and the residual oil had no activity at all (Table II).

The results of chromatographic separation showed maximum activity in the fraction eluted with chloroform. The activity of this fraction is shown in Tables Nos. III and IV.

TABLE III.—ACTIVITY OF CHLOROFORM ELUTED FRACTION BY THE AGAR CUP PLATE ASSAY

Fraction	Dilution	Diameter of Zone of Inhibition in mm. Against *M. aureus*
Eluted with chloroform	1:1000	25.5
	1:2000	23.0
	1:4000	17.5

Attempts were made to crystallize the active principle with different solvents but without success. The material was viscous, sticky, and probably hygroscopic in nature. Hence, carbon, hydrogen and oxygen analyses were not carried out.

The ultraviolet absorption spectrum of this material in ethyl alcohol (2.3 mg./100 cc. of ethyl alcohol) was taken in the range from 220 mμ to 320 mμ (see Fig. 2). The spectrum was compared with that reported by Ormancey-Potier, *et al.* (1), for calophyllolide, calophyllic acid, hydroxy acid of calophyllolide, and tetrahydrocalophyllic acid.

TABLE IV.—ACTIVITY OF CHLOROFORM ELUTED FRACTION OF UNDI OIL

Assay	Active Dilution		
	M. aureus	*B. subtilis*	*M. albus*
Agar streak	1:40,000	1:50,000	1:50,000
Serial dilution	1:22,000	1:34,000	1:34,000

The active principle obtained by us from Undi oil shows a maximum at 312 mμ and a minimum at 272 mμ. This does not resemble any of the observations made by Ormancey-Potier *et al.* (1) on certain products isolated from the defatted kernels of *Calophyllum inophyllum*. However, the shape of the curve obtained by us resembles that of tetrahydrocalophyllic acid. The difference between the maximum and minimum for their product works out to 42, whereas the difference between maximum and minimum for the active principle obtained by us works out to 40. It may be that this material is a new product or it may be a homolog of tetrahydrocalophyllic acid since the difference between the maxima and minima for these products is nearly the same. The raw material used by Ormancey-Potier *et al.* (1), was from Madagascar.

SUMMARY

1. Undi oil obtained by different methods of extraction showed antibacterial activity of the same order.

2. A preliminary screening test showed that the oil was active against several Gram-positive organisms tested.

3. The active principle was stable to autoclaving and at room temperature for one month in the form of emulsion. It could be completely extracted by 90% v/v ethyl alcohol. The activity of the extract was 14 times that of the original oil against *M. aureus* by the agar cup plate method.

4. The separation of the active principle was studied by molecular distillation and chromatography. Maximum activity was shown by a fraction adsorbed on alumina and eluted with chloroform.

The chromatographically separated fraction has been characterized from its ultraviolet absorption.

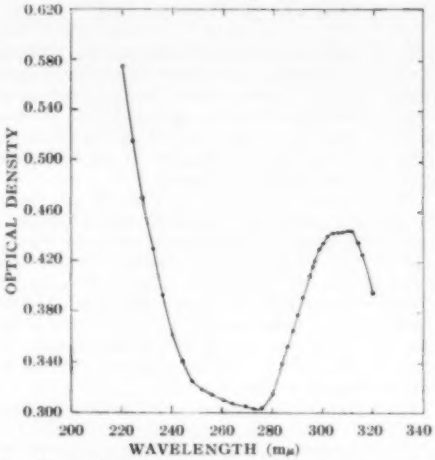

Fig. 2.—Ultraviolet absorption spectrum of the active principle from Undi oil.

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Some Attempts to Prolong the Duration of Action of 2,2-Diethyl-1,3-Propanediol (Prenderol®)*

By IRWIN H. SLATER, JOHN F. O'LEARY, and E. J. PRIBYL

Although 2,2-diethyl-1,3-propanediol (Prenderol®) is a compound of pharmacologic interest, it is difficult to use clinically because of its short duration of action. A series of 23 compounds related to 2,2-diethyl-1,3-propanediol and 2,2-diethyl malonic acid have been screened for anticonvulsant activity in mice in an attempt to find a drug with a longer duration of action. Of these, diethyl malonyl diurethane was the most interesting since it protected mice from the lethal and convulsant action of pentylenetetrazol for several hours. This effect was observed at doses which caused little observable change in the behavior of the mice. Higher doses caused clonic convulsions.

AMONG the new compounds of possible use in the management of convulsive and spastic disorders, 2,2-diethyl-1,3-propanediol (DEP, Prenderol®) appears to be of considerable pharmacologic interest, but it is difficult to use clinically because of its brief duration of action. Initial clinical studies have indicated that although the drug shows evidence of favorable effects in spasticity and in petit mal epilepsy, large and frequent doses are needed to maintain the effect [see Schwartz and Slater (1), Denhoff, *et. al.* (2), Gibbs and Stamps (3).]

The central nervous system activity of this compound was noted by Lott (4) in a study of a series of mephenesin analogs. Berger (5) called attention to the striking effectiveness in the protection of mice given convulsant doses of pentylenetetrazol (Metrazol®). More recent work on spinal cord reflexes, on facilitory and inhibitory activity of the brainstem, and on the electroencephalogram has indicated that Prenderol® is a depressant and anticonvulsant somewhat similar to trimethadione in pharmacologic properties. In contrast to trimethadione, however, Prenderol® is metabolized rapidly. Berger and Ludwig (6) have reported that in man, 2,2-diethyl-1,3-propanediol undergoes an oxidation of one terminal hydroxyl group to an inactive acid compound, diethyl hydracrylic acid. These workers attempted to prolong the effectiveness of the compound by preparing esters which would hydrolyze slowly in the body. Neither the disuccinate, dibenzoate, monoacetate, nor the diacetate seemed particularly successful, although the diacetate did show a duration of action somewhat longer than Prenderol®. More recently, Berger has published the results of studies on a series of monocarbamate and dicarbamate esters of various 2,2-disubstituted 1,3-propanediols. Of these the dicarbamates were found to have a longer duration of action and a higher potency than the corresponding diols.

This study which was initiated prior to the publications of Berger and his co-workers (7) likewise was directed at attempts to prolong the duration of action of Prenderol®. We have investigated a series of compounds which are in a sense derivatives of diethyl propanediol in which the terminal CH_2OH groups have been modified. These include structures related to the diethyl derivatives of 1,3-propanediol, 1,3-diamino-propane, and malonic acid. The anticonvulsant potency and duration of action of these compounds have been studied in mice.

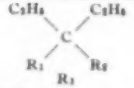
EXPERIMENTAL

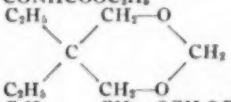
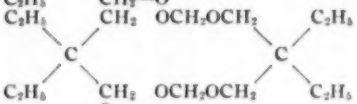
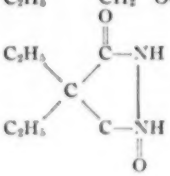
Methods.—The compounds were tested on Lynch strain mice, five to seven weeks of age weighing 16–22 Gm. The ED_{50} refers to the dose at which the righting reflex was lost for more than one minute. The medium protective doses against the lethal and convulsant effects of twice the median lethal dose of pentylenetetrazol (PD_{m50}) or strychnine (PD_{s50}), given intraperitoneally at the time of maximal effect of the drug, was determined. PD_e is the median protective dose against the extensor thrust phase of supramaximal electroshock seizure elicited by 60 c./sec. current of twice the amperage necessary to cause extensor thrust in one-half the animals. This was determined at 10, 40, 70, and 100 minutes after the intraperitoneal injection of the drug. Duration is expressed as the time at which more than half of the animals were still protected after a dose which had been sufficient to protect all at the time of maximal effect. This represents a crude indication of the duration of action of the drug.

Since it did not seem practical to determine the concentration of the drug chemically, we have tested the duration of the action of the drug after oral administration by measuring the PD_{m50} at 20, 60, 120, 300 and 420 minutes. The assay was terminated when the dose necessary to protect the mice caused demonstrable disturbance in the behavior of the animals (i. e., ataxia, sleep, excitement, or convulsions). The median effective dose was deter-

* Received November 30, 1953, from the Division of Orthopaedic Surgery, Department of Surgery and Division of Pharmacology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y., and the Squibb Institute for Medical Research, New Brunswick, N. J.

TABLE I.—EFFECTIVENESS OF THE COMPOUNDS IN MM/KG. AFTER INTRAPERITONEAL INJECTION



No.	MC	R_1	R_2	LD_{50}	ED_{50}	$PD_{50,60}$	$PD_{50,30}$	Duration, Min.
1	1415	CH_2OH	CH_2OH	10.0 ± 1.4	2.66 ± 4.7	1.21 ± 3.6	1.16 ± 3.6	10
2	2579	CH_2OOCCH_2	CH_2OOCCH_3	6.9 ± 11	4.5 ± 12	1.1 ± 18	0.94 ± 14	40
3	2550	$CH_2OCOCH_2CH_2COONa$	CH_2OH	11.3	6.7	3.0	3.8	100+
4	3408	$CH_2OCONHCONH_2$	CH_2OH	>9.6	.....	.....	1.3	..
5	2679	$CH_2OCON(CH_3)_2$	CH_2OH	3.0 ± 6	1.5 ± 3	>2.1	0.6 ± 8	10
6	2690	$CH_2OCON(CH_3)_2$	$CH_2OCON(CH_3)_2$	2.1 ± 14	Convulsant	>1.0	1.2 ± 24	70
7	2705	$CH_2OCOCH_2N(C_2H_5)_2$	$CH_2OCOCH_2N(C_2H_5)_2$	>7.4	4.5	2.1	2.0	10
8	2340	$CHOH\text{-}C_6H_5$	CH_2OH	1.4 ± 12	0.9 ± 12	0.6 ± 8	1.2	70
9	2339	$CHOH\text{-}C_6H_5$	$COOC_2H_5$	2.7 ± 11	1.4 ± 5	>1.1	1.0 ± 9	10
10	2666	$CH(OC_2H_5)_2$	CH_2OH	5.5 ± 9	.....	>3	>2	..
11	2602	$CONH_2$	$CONH_2$	>13.2	>13.2	>13.2	>13.2	..
12	2667	$CONHCH_2OH$	$CONHCH_2OH$	>9.6	>9.6	>9.6	>9.6	..
13	2659	$CONHCOOC_2H_5$	$CONHCOOC_2H_5$	3.2 ± 8	Convulsant	0.4 ± 15	0.77 ± 15	100+
14	3024	$CH_2NHCOOC_2H_5$	$CH_2NHCOOC_2H_5$	2.4 ± 15	0.78 ± 18	>0.6	>0.6	..
15	3043	$CH_2NHCONH_2$	$CH_2NHCONH_2$	>9.7	>9.7	>2.9	>2.9	..
16	3072	$CH_2NHCON(CH_3)_2$	$CH_2NHCON(CH_3)_2$	0.016 ± 9.1	.....	>0.01	>0.01	..
17	3504[a]	$CONHCH_2CH_2CH_2OCH_3$	$CONHCH_2CH_2CH_2OCH_3$	>7.7	>7.7	>7.7	>7.7	..
18	3198[b]	$CONHCOOC_2H_5$	$CONHCOOC_2H_5$	>8.5	>8.5	5.5	6.4	100+
19	2446			5.1 ± 5	3.5 ± 6	1.8 ± 12	3.0 ± 12	100
20	2445			>9.2	>9.2	>9.2	>9.2	..
21	2964			>13.5	>13.5	>13.5	>13.5	..

22	3511			1.9 ± 25	Convulsant	>0.22	>0.22
23	3613		$N{-}CH_2CH_2N(C_2H_5)_2 \cdot HCl$	0.27 ± 17	Convulsant	>1.2	>1.2
24	2778		$CaCl_2$	2.50 ± 15	1.4	0.6	1.0

[a] 3504 is the derivative of dimethyl malonic acid.

[b] 3198 is the derivative of malonic acid (unsubstituted). SE in %.

mined by the method of Miller and Tainter. For convenience of comparison all doses are expressed in millimoles per kilogram body weight. Some of the compounds which appeared to be of little interest on the basis of the initial intraperitoneal evaluation were not assayed orally.

Results.—The results of the initial intraperitoneal screening are summarized in Table I. In Table II we have evaluated the duration of the effectiveness of the more promising of these compounds when administered by the oral route. PD_{50} is omitted from the table since none of the compounds was particularly potent by this test. Modification of the terminal CH_2OH groups resulted in changes in the potency, duration, and character of the pharmacologic activity of the compounds.

In mice Prenderol® usually causes pre-anesthetic excitement; some of these compounds caused little or no excitement while others were convulsants. The nature of the effectiveness of the various drugs as anticonvulsant agents also attests to the differing mode and site of action.

Compounds 2 and 3, the diacetate and the monosuccinate were similar to Prenderol® in mode of action. After intraperitoneal injection the duration of action was somewhat longer and the potency somewhat lower for these compounds, particularly for the succinate. After oral administration, however, these differences were less marked. This suggests that a significant amount of hydrolysis to Prenderol® occurs prior to absorption of the drugs from the gastrointestinal tract. Compound 4, the allophanate of Prenderol® showed predominantly hypnotic activity and was not examined in detail.

The monodimethylcarbamate was potent, especially against the extensor phase of supramaximal electroshock, but had a short duration of action. The *bis*-dimethylcarbamate was a convulsant which was somewhat effective against electroshock but still of relatively short duration of action. Compounds 8, 9, and 10 in which substitutions were made on the terminal carbon atoms of Prenderol® were hypnotic in mice but had a short duration of action.

Of the compounds studied, number 13 (MC 2659) diethyl malonyl diurethane (N,N′-dicarbethoxy-α,α-diethylmalonamide) seemed to be the most promising. At the end of seven hours, the majority of the animals given an initial dose of 280 mg./Kg. by mouth were still protected against two lethal doses of Metrazol®. This is the only compound in the group which seems to surpass trimethadione in duration. This compound caused only slight ataxia and increased activity at doses sufficient to cause this long lasting protection against the lethal and convulsant effects of Metrazol®. At higher doses MC 2659 caused clonic convulsions quite different in character from the tremorous seizure one sees with high doses of hydantoins and thiohydantoins. Some pilot experiments in rats and cats indicate that the convulsive activity of the compound can be demonstrated in these animals as well. Whether this type of convulsive activity would represent a serious drawback to the possible clinical usefulness of this drug cannot be determined solely on the basis of these experiments in mice. To date we have been unable to develop a convenient and reliable method for the synthesis of this compound and with the small amounts available have not undertaken any detailed neuropharmacologic inves-

TABLE II.—ORAL PROTECTIVE DOSE AGAINST PENTYLENETETRAZOL IN MM/KG. AFTER VARIOUS TIME INTERVALS

		Time, Min.					
		20	60	120	210	300	420
Trimethadione		4.85	3.66	4.78	5.27	7.03	9.75
1	1415 (DEP)	1.6	2.6	3.8	4.7		
2	2579	1.4	3.6	4.5	4.0		
3	2550	4.2	6.3	7.4	9.6	11.3	
5	2679	3.9	4.0	4.9			
6	2690	3.4	3.4	3.4			
8	2340	1.3	2.0	3.5			
11	2602	13.2	13.2	13.2			
12	2667	14.6	14.6	14.6			
13	2659	..	0.55	0.61	0.55	0.61	0.76
19	2446	3.9	3.5	4.5	5.8	6.0	
25	2778	0.8	1.1	1.8	2.5		

tigation of it. This compound has been described previously by Traube (8).

The unsubstituted malonyl diurethane (compound 18) and bis-(3-methoxypropyl)-dimethylmalonamide (compound 17) were devoid of activity at the levels tested.

Compounds 14 and 15 were without anticonvulsant activity. Compound 16 caused a pattern activity seen with peripheral blocking agent. It appears to be a ganglionic blocking drug.

Of the cyclic derivatives considered, 5,5-diethyl-1,3-dioxane (compound 19) showed significant prolongation of anticonvulsant effect. This compound caused considerable drowsiness at the higher dose levels necessary for prolongation of the effect through the sixth hour. Compounds 22 and 23 were both convulsants which unlike MC 2659 did not show any anticonvulsant activity at the various dose levels tested. There was no prolongation of duration of action with compound 24.

SUMMARY

1. A series of derivatives of 2,2-diethyl-1,3-propanediol (Prenderol®, DEP) and 2,2-diethyl malonic acid were screened for anticonvulsant activity in mice in an attempt to find a compound of longer duration of action than Prenderol®.

2. Of the compounds studied diethyl malonyl diurethane was the most interesting. When given in amounts which cause little change in the activity or behavior of mice, diethyl malonyl diurethane is effective in preventing the convulsant and lethal effects of pentylenetetrazol for a period of six to eight hours. At high dose levels in mice, rats, and cats, this compound causes clonic convulsions.

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WHO MAKES IT?

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Tri(*p*-nitrophenyl)carbinol
Piperidine-4-acetic acid
2,3,5,6-Tetramethylbenzoic acid
Succindialdehyde
4-Nitrohydrindene
2,4-Dichlorobenzyl alcohol
1,2,3-Butanetriol
Phthalazone
Reductone
Mucinase
Cyclital
Cymarin
γ-Tocopherol

The Electrolytic Effects of Licorice Preparations in the Adrenalectomized Rat*

By WILLIAM E. HASSAN, Jr.,† JOSEPH F. PALUMBO,‡ and FRED ELMADJIAN§

Spanish licorice extract, ammoniated glycyrrhizin, monoammonium glycyrrhizinate and glycyrrhetinic acid were tested for electrolytic effects similar to those of desoxycorticosterone acetate. All the compounds tested, except glycyrrhetinic acid, produced electrolytic changes when administered orally to adrenalectomized rats. All fractions, except glycyrrhetinic acid, produced a K/Na ratio which with increased drug dosage resembles that of desoxycorticosterone acetate.

DURING the past ten years a number of reports have appeared in the literature about the use of licorice extract in the treatment of Addisonian patients. All of the reports appearing in the medical journals are the results of clinical observations and tests. To date, no controlled laboratory experiments to determine whether or not licorice extract possesses an electrolytic effect in the adrenalectomized rat have been reported.

The purpose of this investigation was to test biologically by the method of Marcus, Romanoff, and Pincus (1) the various licorice fractions available in order to ascertain by accepted laboratory methods whether or not these fractions had electrolytic activity.

HISTORY

Revers (2) in 1946 investigated the usefulness of licorice extract in the treatment of peptic ulcer. In a later report he (3) noticed that approximately 20 per cent of his patients developed edema, hypertension, or cardiac asthma or both. It was clearly evident that these symptoms could not have been due to cardiac or renal complications for it was stated that the electrocardiograms were normal, that there was no change in the urine, and that there was no diminution in serum protein content.

Borst, *et al.* (4), and Molhuysen, *et al.* (5), in 1950, demonstrated that extract of licorice exerted on human beings a biological activity which simulated that of desoxycorticosterone in so far as the electrolyte balance of sodium and potassium is concerned.

In 1951 Groen *et al.*, (6), confirmed the results obtained by Borst and Molhuysen. In an addendum to their paper it was stated that the daily administration of 3.3 Gm. of crude ammonium glycyrrhizinate kept a patient, previously maintained on 2.5 mg. desoxycorticosterone acetate daily, in sodium and potassium equilibrium.

Late in 1951 Groen, *et al.* (7), published a report that includes an observation on the effect of glycyrrhizinic acid on the electrolyte metabolism in Addison's disease. In the summary of this paper the following statement was made: "A case of Addison's disease is described in which, after the administration of desoxycorticosterone was stopped, electrolyte equilibrium was maintained by the daily administration of 3–4 Gm. of ammonium glycyrrhizinate by mouth. On withdrawal of the glycyrrhizinate administration the patient responded by sodium and chloride loss, potassium retention, and clinical signs of dehydration and hemoconcentration. Administration of crude licorice extract brought the electrolyte metabolism to normal. This observation seems to prove that the glycyrrhizinic extract of licorice exerts its influence on the electrolyte metabolism in Addison's disease."

METHOD

Male albino rats of the Wistar strain weighing between 135 and 150 Gm. were used. Each rat was bilaterally adrenalectomized under ether anesthesia and was maintained on Purina Chow diet. Physiological saline solution was used for drinking water. Cook and Elmadjian (8) used two-day and three-day adrenalectomized rats. Since they have shown that best results were obtained on these days we have also used two-day and three-day adrenalectomized rats. Food and saline were withheld for three hours prior to the experiments.

At the time of the experiments the rats were etherized to insure complete bladder emptying. The rats were then injected as indicated with aqueous solutions of the following: sodium chloride (0.9%), Spanish licorice extract, monoammonium glycyrrhizinate, ammoniated glycyrrhizin, and glycyrrhetinic acid orally, and with the oily solution of desoxycorticosterone acetate parenterally. Peanut oil was used as the vehicle for the desoxycorticosterone acetate. The remainder of the experimental procedure is similar to that described by Cook and Elmadjian (8).

The K/Na ratio for each fraction is determined by calculating the ratio for the individual pair of animals in the assay at any one dosage level. The ratios so obtained are then averaged and this figure is recorded as the K/Na ratio for the entire group

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The authors wish to acknowledge gratefully the cooperation of C. K. Swift of MacAndrews and Forbes Company in providing the following materials which were used throughout this study: ammoniated glycyrrhizin, Spanish licorice extract ref. anal. No. 3902 A, monoammonium glycyrrhizinate batch No. 16-592 B, and glycyrrhetinic acid batch No. 16-552 M.

TABLE I.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF 5-CC. NORMAL SALINE SOLUTION AND DCA SUBCUTANEOUSLY IN ADRENALECTOMIZED RATS[a]

Dose of DCA	No. Pairs per Assay	Urine Volume, cc.	Sodium mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
Control	14	6.1	1.86	±0.12	1.01	±0.03
6 μg.	8	5.1	1.49	±0.36	1.39	±0.14
12.5 μg.	8	4.6	1.06	±0.13	1.15	±0.16
25 μg.	8	3.1	0.74	±0.03	1.54	±0.18
50 μg.	15	4.6	0.96	±0.07	1.40	±0.06

[a] (8) Cook, M. R., and Elmadjian, F.

of assays at that particular dosage level. These ratios are recorded for each fraction in Table VI.

In this work 0.70 was set as an arbitrary ratio to indicate the lowest range of activity. In other words, any dosage level producing a ratio of less than 0.70 is to be considered inactive; and any dosage level showing a ratio of 0.70 or over is to be considered as active. In this way the activity produced by the dose of the drug can be compared with that of desoxycorticosterone acetate.

DISCUSSION

Previous workers, (1) and (8), have shown that desoxycorticosterone acetate caused a retention of urine, a decreased output of sodium ion, and an increased output of potassium ion in the urine. They have also demonstrated that the method of assay employed was sensitive to activity in doses as low as 6 μg. desoxycorticosterone acetate. Therefore it was reasonable to assume that if 5 cc. of physiological saline solution and doses of licorice extract or its fractions were administered to two-day and three-day bilaterally adrenalectomized rats, a decrease in urine and sodium output and an increase in potassium output would indicate that the licorice extract possessed an electrolytic activity. Furthermore, Groen, *et al.* (6), stated that licorice extract and crude ammonium glycyrrhizinate produced a desoxycorticosterone-like activity when administered to human beings orally, a distinct advantage over the desoxycorticosterone acetate which must be administered parenterally.

A survey of the results as presented in Tables II–VI shows that two of the three criteria that indicate activity are present: namely, the decreased urine and sodium output. The potassium ion instead of being excreted was being withheld in approximately the same proportion as the sodium ion. Therefore, since the mere comparison of these figures did not seem to indicate biological activity similar to that of the desoxycorticosterone acetate, another method of expressing the results has been employed.[1] Other workers (9) have shown that the sodium potassium ratio is more sensitive to DCA-like activity than the change in the excretion of each ion taken by itself. According to this method of ratio expression increased DCA-like activity is reflected by a decrease in the ratio. It was therefore felt that a simpler method of ratio expression was desirable in order to show an increased ratio commensurate with increased activity of the fraction administered. This was done by merely expressing the ratio as the potassium sodium ratio (K/Na).

RESULTS

Spanish Licorice Extract.[2]—The results obtained by the use of Spanish licorice extract are tabulated in Table II. A comparison of these data with that of the control animal data in Table I clearly indicates that this fraction induced a retention of urine and of sodium ion, particularly at the 500-mg. and 1,000-mg. dose levels. Excretion of the potassium ion was not increased significantly. In the high dose level of 2,000 mg. per animal marked diarrhea developed, which contaminated the urine specimens. Reference to Table VI, which shows the K/Na ratio, indicates that 500-mg., 1,000-mg., and 2,000-mg. doses of Spanish licorice extract produce a K/Na ratio far above 0.70.

Ammoniated Glycyrrhizin.—Table III contains experimental data obtained by the oral administration of ammoniated glycyrrhizin to adrenalectomized rats. Once again two of the three criteria indicative of DCA-like activity are present: namely, a significant decrease in sodium output and a tendency toward retention of urine. Reference to Table VI

[1] The calculation is described under section on *Methods*.
[2] Powdered extract; 4% moisture content.

TABLE II.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF BOTH 5-CC. NORMAL SALINE SOLUTION AND SPANISH LICORICE EXTRACT IN ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
90	24	2.2	1.68	±0.12	1.56	±0.09
180	15	4.6	1.66	±0.22	1.03	±0.03
270	10	4.7	1.78	±0.22	0.87	±0.06
500	8	3.3	1.34	±0.43	1.05	±0.36
1000	8	2.8	0.90	±0.12	0.95	±0.07
2000	6	4.0	1.69	±0.38	1.43	±0.41

TABLE III.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ADMINISTRATION OF AMMONIATED GLYCYRRHIZIN IN 5-CC. NORMAL SALINE SOLUTION ORALLY TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	21	3.9	1.27	±0.09	0.81	±0.06
100	14	3.7	1.42	±0.13	0.84	±0.06
250	18	4.7	1.55	±0.11	0.82	±0.04
500	14	3.7	1.24	±0.14	0.89	±0.05
750	7	4.7	1.35	±0.09	1.11	±0.05
1000	18	3.9	0.85	±0.09	0.68	±0.05

TABLE IV.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF PURE MONOAMMONIUM GLYCYRRHIZINATE IN 5-CC. NORMAL SALINE SOLUTION TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 Gm.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	21	2.9	0.95	±0.07	0.49	±0.04
75	14	2.9	1.06	±0.13	0.67	±0.23
100	14	5.0	1.13	±0.05	0.82	±0.22
150	10	4.7	1.17	±0.06	1.04	±0.02
200	18	6.3	1.39	±0.03	1.18	±0.06
250	21	3.6	1.19	±0.07	0.66	±0.04

indicates that doses of 500 mg up to 1,000 mg. of the licorice fraction (ammoniated glycyrrhizin) produce a K/Na ratio above 0.70.

Monoammonium Glycyrrhizinate.—Table IV contains the experimental data obtained by the oral administration of graded doses of pure monoammonium glycyrrhizinate to adrenalectomized animals. Once again the urine retention and decreased sodium output properties of the compound are evident. Table VI shows that 100 mg. of this salt produced a K/Na ratio of 0.75.

It is interesting to note at this point the activity relationship of the three fractions discussed thus far: Spanish licorice extract and ammoniated glycyrrhizin started to exhibit activity at the 500-mg. dose level, whereas monoammonium glycyrrhizinate produces the same approximate comparative activity with one-fifth of the dose.

Glycyrrhetinic Acid.—Since the pure monoammonium glycyrrhizinate fraction exhibited the greatest activity, it was anticipated that the glycyrrhetinic acid, prepared by the acid hydrolysis of the monoammonium salt, would produce the ultimate in activity. Reference to Table V and to Table I for the controls, shows that the greatest urine and sodium retention is produced by a dose of 200 mg. of the acid. The potassium also has been retained to a marked degree. Reference to Table VI for the K/Na ratio shows that at this level the ratio is only 0.52, which we have already indicated is inactive. An interesting feature of this experiment is that the relative activity (according to the K/Na ratio) at the 50-mg. dose level steadily decreases with the increased dosage and that this decreased ratio is due to the potassium retention, which is far greater than the sodium retention.

TABLE V.—URINARY EXCRETION OF SODIUM AND POTASSIUM FOLLOWING THE ORAL ADMINISTRATION OF GLYCYRRHETINIC ACID IN 5-CC. NORMAL SALINE SOLUTION TO ADRENALECTOMIZED RATS

Dose in mg.	No. Pairs per Assay	Urine Volume, cc.	Sodium, mg./hr./100 cc.	S. E.	Potassium, mg./hr./100 Gm.	S. E.
50	14	4.3	1.44	±0.16	1.01	±0.07
75	7	4.4	1.54	±0.04	0.91	±0.15
100	14	4.2	1.77	±0.17	0.79	±0.01
200	12	2.8	1.08	±0.01	0.54	±0.05

TABLE VI.—POTASSIUM–SODIUM RATIO, K/Na

DCA		Spanish Licorice Extract		Ammoniated Glycyrrhizin		Monoammonium Glycyrrhizinate		Glycyrrhetinic Acid	
Dose	Ratio	Dose[a]	Ratio	Dose[a]	Ratio	Dose[a]	Ratio	Dose[a]	Ratio
Control	0.54	90	0.67	50	0.66	50	0.52	50	0.74
6 μg.	0.86	180	0.61	100	0.60	75	0.65	75	0.67
12.5 μg.	1.08	270	0.60	250	0.55	100	0.75	100	0.51
25 μg.	2.00	500	0.83	500	0.80	150	0.90	200	0.52
50 μg.	1.40	1,000	1.05	750	0.83	200	0.87		
		2,000	0.83	1,000	1.05	250	0.59		

[a] Dose in milligrams per animal.

When these dosage ranges are compared with what is reported to be effective in man, it is noted that the rat requires relatively large doses. It may be that the rat is not the most suitable animal for determining the biological activity of licorice.

SUMMARY

1. Spanish licorice extract, ammoniated glycyrrhizin, monoammonium glycyrrhizinate and glycyrrhetinic acid were tested for electrolytic activity similar to that of desoxycorticosterone acetate.

2. Spanish licorice extract, ammoniated glycyrrhizin, and monoammonium glycyrrhizinate, administered orally, exhibited an electrolytic activity when this activity is expressed as the K/Na ratio. It was observed that, first, there was an indication of quantitative decrease in sodium output and urine retention with increased drug dosage; second, there was little or no change in the potassium output.

3. Spanish licorice extract, ammoniated glycyrrhizin and monoammonium glycyrrhizinate produce a K/Na ratio which follows that of desoxycorticosterone acetate with increased dosage. Glycyrrhetinic acid does not follow the desoxycorticosterone acetate pattern because of its greater potassium retention effect.

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The Effect of Freeze-Drying on Chlorophyll in the Leaves of Some Selected Drug Plants*

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A comparative study of the effect of freeze-drying and oven-drying at 45–50° on chlorophyll in the leaves of some selected drug plants was undertaken. The results indicated that more total chlorophyll was obtained from the freeze-dried leaves. The major difference was in the amount of chlorophyll *a*. Freeze-drying facilitated total chlorophyll extraction.

WHILE voluminous reports concerning the application of freeze-drying to biological substances are available, very little information concerning the application of freeze-drying as a possible artificial physical means of crude drug drying and preservation could be found in the literature. Chambers and Nelson (1) were the first workers to investigate the use of high vacuum freeze-drying as a means of crude drug preservation. In order to adapt freeze-drying more extensively, further application of the process to other drug plants and their constituents was thought advisable.

The chlorophyll pigments in some selected leaves of drug plants were chosen as the constituents of the investigation, because the leaves of plants readily lose their bright greenish color when dried at elevated temperatures and become brownish after drying and storage. By freeze-drying at a lower temperature range it was thought that less chemical changes and alterations of the chlorophyll would occur, thus preserving the appearance of the leaves.

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The authors gratefully acknowledge the assistance of the Statistics Laboratory of The Ohio State University in evaluating the data listed in Tables I, II, and III.
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EXPERIMENTAL

Collection and Drying of the Leaves.—The leaves of *Podophyllum peltatum*, Linné (podophyllum); *Ricinus communis*, Linné (castor), and *Mentha spicata*, Linné (spearmint) were selected as the material of investigation in this work.

Since chlorophyll is the substance of interest in this investigation, any kind of leaves could have been used, but the three mentioned leaves were chosen because they were available in the drug plant garden at the time of collection, and because, especially, podophyllum and castor leaves are comparatively large. The leaves were collected from the Medicinal Plant Garden of the College of Pharmacy of the Ohio State University, Columbus, Ohio. Each representative sample was divided into two groups. One group was oven-dried at 45–50° and the other group was freeze-dried. After drying, the samples were reduced to a No. 40 powder by means of a Wiley mill.

It was noted that the oven-dried samples of the three kinds of leaves had a darker green color than the freeze-dried samples.

Moisture Determination.—The moisture content of oven-dried and freeze-dried samples of the leaves was determined by the U. S. P. XIV method (2) for drugs containing no constituents volatile at 100°. Freeze-drying removed more moisture from the leaves than oven-drying at 45–50°.

The purpose of performing the moisture determination was to obtain a percentage of moisture from which the succeeding calculations could be compared on a dry weight basis.

Determination of Total Chlorophyll and the *a* and *b* Components.—Total chlorophyll and its *a* and *b*

TABLE I.—DETERMINATION OF TOTAL CHLOROPHYLL AND THE A AND B COMPONENTS OF THE LEAVES OF MENTHA SPICATA, LINNÉ (SPEARMINT) BY SPECTROPHOTOMETRIC METHOD

	Freeze-Dried	Oven-Dried	Difference	Standard Deviation of the Difference	Ratio of Difference to its Standard Deviation
		Total Chlorophyll[a]			
Sample 1	0.4077	0.3850	0.0227	0.0311	0.730
Sample 2	0.4062	0.3782	0.0280	0.0153	1.830
Sample 3	0.4004	0.3667	0.0337	0.0208	1.620
Average	0.4048	0.3766	0.0282	0.0135	2.089[b]
		Chlorophyll *a*[a]			
Sample 1	0.2769	0.2654	0.0115	0.0125	0.920
Sample 2	0.2759	0.2582	0.0177	0.0073	2.425[b]
Sample 3	0.2738	0.2535	0.0203	0.0089	2.281[b]
Average	0.2755	0.2590	0.0165	0.0057	2.895[b]
		Chlorophyll *b*[a]			
Sample 1	0.1317	0.1202	0.0115	0.0211	0.545
Sample 2	0.1312	0.1203	0.0109	0.0112	0.973
Sample 3	0.1268	0.1134	0.0134	0.0153	0.876
Average	0.1299	0.1180	0.0119	0.0095	1.253

[a] Expressed as percentage.
[b] Indicates significant difference.

TABLE II.—DETERMINATION OF TOTAL CHLOROPHYLL AND THE A AND B COMPONENTS OF THE LEAVES OF RICINUS COMMUNIS, LINNÉ (CASTOR) BY SPECTROPHOTOMETRIC METHOD

	Freeze-Dried	Oven-Dried	Difference	Standard Deviation of the Difference	Ratio of Difference to its Standard Deviation
		Total Chlorophyll[a]			
Sample 1	0.5874	0.4905	0.0969	0.0210	4.614[b]
Sample 2	0.5920	0.4984	0.0936	0.0453	2.066[b]
Sample 3	0.6048	0.4986	0.1062	0.0229	4.638[b]
Average	0.5947	0.4958	0.0989	0.0183	5.404[b]
		Chlorophyll *a*[a]			
Sample 1	0.4387	0.3571	0.0816	0.0107	7.626[b]
Sample 2	0.4421	0.3569	0.0852	0.0225	3.787[b]
Sample 3	0.4530	0.3574	0.0956	0.0153	6.248[b]
Average	0.4446	0.3751	0.0695	0.0097	7.165[b]
		Chlorophyll *b*[a]			
Sample 1	0.1487	0.1334	0.0153	0.0259	0.591
Sample 2	0.1499	0.1415	0.0084	0.0323	0.260
Sample 3	0.1518	0.1412	0.0106	0.0196	0.541
Average	0.1501	0.1387	0.0114	0.0153	0.754

[a] Expressed as percentage.
[b] Indicates significant difference.

TABLE III.—DETERMINATION OF TOTAL CHLOROPHYLL AND THE A AND B COMPONENTS OF THE LEAVES OF PODOPHYLLUM PELTATUM, LINNÉ (PODOPHYLLUM) BY SPECTROPHOTOMETRIC METHOD

	Freeze-Dried	Oven-Dried	Difference	Standard Deviation of the Difference	Ratio of Difference to Its Standard Deviation
		Total Chlorophyll[a]			
Sample 1	0.4500	0.4073	0.0427	0.0994	0.430
Sample 2	0.4548	0.4102	0.0446	0.0728	0.613
Sample 3	0.4615	0.3748	0.0867	0.0610	1.421
Average	0.4554	0.3974	0.0580	0.0458	1.266
		Chlorophyll *a*[a]			
Sample 1	0.3089	0.2743	0.0346	0.0265	1.306
Sample 2	0.3096	0.2680	0.0416	0.0240	1.733
Sample 3	0.3161	0.2451	0.0710	0.0141	5.035[b]
Average	0.3115	0.2624	0.0491	0.0128	3.836[b]
		Chlorophyll *b*[a]			
Sample 1	0.1411	0.1330	0.0081	0.0772	0.105
Sample 2	0.1452	0.1422	0.0030	0.0527	0.057
Sample 3	0.1454	0.1297	0.0157	0.0536	0.293
Average	0.1439	0.1350	0.0089	0.0359	0.248

[a] Expressed as percentage.
[b] Indicates significant difference.

components were determined according to the A. O. A. C. method (3). The results are listed in Tables I, II, and III. The percentages are mean values of a minimum of 12 determinations. The results were statistically evaluated by the Statistics Laboratory of The Ohio State University. The hypothesis tested was that the means of the two compared samples, oven-dried and freeze-dried, are the same. A ratio value greater than 1.950, obtained by dividing the mean difference by the standard deviation, was considered significant, since according to a table of normal distribution (4) the chances of obtaining a greater value is less than 5%.

The Rate of Chlorophyll Extraction.—When extracting chlorophyll from the leaves, a greater difficulty was encountered and more time consumed in extracting the oven-dried samples as compared with the freeze-dried samples. The object of running the extraction studies was therefore to substantiate this finding by numerical data.

A Fisher electrophotometer, AC Model, was first calibrated according to the A. O. A. C. method (3), with 1.0 cm. absorption cells and a No. 650(A) light filter. A calibration curve that rigidly followed Beer's law was constructed. This was employed for calculating the data shown in Table IV.

The extraction procedure followed was as follows: about 5 Gm. accurately weighed, of each of the oven-dried and freeze-dried powdered material of all the three leaf samples were packed lightly in 80-cc. percolators of the same size and diameter. The powders were moistened with 35 cc. of 85% acetone C. P., and when the liquid was about to drip from the percolators, the lower orifice was closed and the drugs were allowed to macerate for twenty-four hours. The percolation was then allowed to proceed at a moderate rate, gradually adding a new

TABLE IV.—THE RATE OF EXTRACTION OF TOTAL CHLOROPHYLL FROM PODOPHYLLUM, CASTOR, AND SPEARMINT LEAVES[a]

Successive 50-cc. Fractions	Total Chlorophyll in mg./L.					
	Podophyllum Leaves		Castor Leaves		Spearmint Leaves	
	Oven-Dried	Freeze-Dried	Oven-Dried	Freeze-Dried	Oven-Dried	Freeze-Dried
1	260.0	330.0	285.0	360.0	230.0	280.0
2	6.2	3.8	9.0	18.0	10.0	15.0
3	3.5	1.6	4.8	9.0	7.0	7.4
4	2.0	0.8	3.4	6.6	6.5	5.2
5	1.8	0.5	2.8	6.4	5.3	3.8
6	1.6	0.4	2.5	5.4	5.2	3.2
7	1.5	0.3	1.7	4.4	4.4	2.0
8	1.4	0.3	1.5	3.4	4.0	1.4
9	1.2	0.2	1.3	3.0	3.9	1.4
10	1.0	0.2	1.3	2.8	3.6	1.2
11	...	...	1.1	2.5	2.6	0.8
12	...	...	1.1	2.3	1.8	0.4
13	...	...	1.0	2.0	1.8	0.4
Total	280.2	338.1	316.5	425.0	286.1	322.2

[a] The results are the average of three determinations.

menstruum in 25-cc. portions to the percolators. Successive 50-cc. fractions of percolate were collected, their per cent transmission immediately determined on the Fisher electrophotometer, AC Model, and the chlorophyll content of each fraction read directly on the calibration curve. Percolation was stopped in each case when the transmission of the last fractions of percolates approached minimum. The results of the extraction studies of chlorophyll in the three kinds of leaves appear in Table IV.

DISCUSSION

In reference to Tables I and II (castor and spearmint leaves) it can be observed that there is more total chlorophyll in the freeze-dried samples than in the oven-dried samples. This difference is a significant amount. The major difference lies in the amount of chlorophyll *a* in the two samples since this difference is of a significant magnitude, while the difference in chlorophyll *b* is not a significant amount.

In the case of podophyllum leaves, as can be observed in Table III, there is an unusual result. The experiment showed no significant difference between the total chlorophyll of the freeze-dried and oven-dried samples. However, the amount of chlorophyll *a* in the freeze-dried sample was significantly greater than in the oven-dried sample. This result can be attributed to several apparent factors. The podophyllum leaves were the first leaves of the experiment to be assayed, and the laboratory technique involved in the experiment was improved in the latter experiments. This fact is evident from the larger standard deviation for the podophyllum experiment in comparison to the standard deviation of the other experiments. The fact that the ratio of chlorophyll *a* to chlorophyll *b* in podophyllum leaves is approximately 2 to 1 while in the castor plant is approximately 2.8 to 1 is another factor. Although the difference in chlorophyll *a* is significant, the large amount of chlorophyll *b*, which does not show a significant difference, offsets somewhat the difference in chlorophyll *a*. It is true that the ratio of chlorophyll *a* to chlorophyll *b* in spearmint leaves is also approximately 2 to 1, and that the large amount of chlorophyll *b* did not offset the difference in chlorophyll *a* enough to keep the difference in total chlorophyll from being significant. But there is a difference in the two cases of podophyllum leaves and spearmint leaves. In podophyllum leaves the amount of chlorophyll *b* in the two compared samples, freeze-dried and oven-dried leaves, is almost the same while in the case of spearmint leaves the freeze-dried sample has more chlorophyll *b* than the oven-dried, even though it is not a significant amount.

In summary, the total chlorophyll results were possibly influenced by the large standard deviation, the high proportion of chlorophyll *b* to chlorophyll *a* and the fact that there was a very minute difference in the amount of chlorophyll *b* in the two compared samples.

The optimum temperature of chlorophyllase, an enzyme that occurs alongside its substratum "chlorophyll" in the green parts of all the plants, is about 20° (5). Either an increase or a decrease in the temperature will retard the activity of the enzyme. Since less chlorophyll was retained in the oven-dried samples than in the freeze-dried samples of all the three leaves, it is apparent that enzymatic activity was retarded to a greater extent in the freeze-dried samples than in the oven-dried ones, hence the less destruction and breakdown of chlorophyll in the freeze-dried samples.

The ratio of chlorophyll *a* and *b* in the oven-dried samples was lower than the ratio of these two components in the freeze-dried samples of the leaves. This, of course, is due to the fact that chlorophyll *a* was more sensitive to oven-drying than freeze-drying temperatures and chlorophyll *b* was equally sensitive to the temperatures of both drying procedures. The ratio of chlorophyll *a* to *b* in the leaves investigated, both oven-dried and freeze-dried, compare favorably with the ratio of these two components as reported by other investigators.

That chlorophyll is extracted more rapidly from the freeze-dried samples than the oven-dried samples of the three kinds of leaves is apparent from the data obtained in Table IV. This may possibly be attributed to the fact that high temperatures caused more shrinkage of the tissue cells than low temperatures, thus exposing less area to the effect of the menstruum.

CONCLUSIONS

The leaves of *Podophyllum peltatum*, Linné (podophyllum), *Ricinus communis*, Linné (castor), and *Mentha spicata*, Linné (spearmint) were used in this investigation. The results showed that:

1. More total chlorophyll was obtained from the freeze-dried leaves of podophyllum, castor, and spearmint than from the oven-dried leaves.
2. The total difference was largely due to the difference in chlorophyll *a* since the difference was of a significant magnitude, whereas the difference in chlorophyll *b* was not a significant amount.
3. The ratio of chlorophyll *a* to *b* in the oven-dried samples was lower than the ratio of these two pigments in the freeze-dried samples of the leaves. The ratio of chlorophyll *a* to *b* in the leaves investigated, both oven-dried and freeze-dried, compares favorably with the ratio of these two components as reported by other investigators.
4. Freeze-drying facilitated chlorophyll extractions.

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Water-in-Oil Emulsifying Agents*

VI. Esters Synthesized from Cholesterol Derivatives

By E. L. CATALINE,† JOSEPH E. SINSHEIMER,‡ and LEE WORRELL§

Fifteen normal esters of 3α-carboxy-Δ⁵-cholestene, thirteen of which were previously unreported, have been prepared. Nine, previously unreported, normal esters of 3α-(hydroxymethyl)-Δ⁵-cholestene have been prepared. The 12-hydroxystearic acid ester and the 9,10-dihydroxystearic acid ester of this carbinol have also been prepared. Cholesteryl 12-hydroxystearate has been prepared.

IN PREVIOUS communications from this laboratory (1–3) the effect of the fatty acid esters of cholesterol in potentiating the emulsifying efficiency of solutions of cholesterol in white petrolatum has been described. In an attempt to gain further insight into the relationship of structure to potentiation of emulsifying efficiency, the following series of esters have now been synthesized: (*a*) esters of 3α-carboxy-Δ⁵-cholestene, (*b*) esters of 3α-(hydroxymethyl)-Δ⁵-cholestene, and (*c*) hydroxystearic acid esters. The evaluation of these compounds will be described in the next paper of this series (4).

Marker, *et al.* (5–7), in their original synthesis of 3-carboxy-Δ⁵-cholestene believed they had isolated a mixture of the two epimers and called the compound *dl*-3-carboxy-Δ⁵-cholestene. Baker and Squire (8) pointed out indications that the acid was actually of one configuration. Then in 1951, Baker and Petersen (9) were able to establish the configuration of this acid to be of the *alpha* series of steroids, that is, of the opposite configuration at carbon number three to that of cholesterol. In the course of this proof, cholesteryl carbinol was synthesized and its configuration was also established as belonging to the *alpha* series. By deduction, since there is no disturbance of the bonds about the asymmetric carbon number three in the esterification of either the acid or the carbinol, the esters formed are also of the *alpha* configuration.

Previous work (10, 2) has indicated that the presence of a hydroxyl group in an ester would increase even further the potentiation of emulsification by the ester. This indication was based upon the observations of such effects by the 12-hydroxystearic acid esters of cetyl alcohol and of cholesterol. While the cetyl ester was isolated in crystalline form, this was not true of the cholesteryl ester.

The problem of obtaining a crystalline product is undoubtedly due to the presence of both a secondary hydroxyl group in the acid and also in cholesterol. The reaction conditions necessary to obtain the cholesteryl ester also led to the esterification of the hydroxyl group of the acid. The self-esterification of 12-hydroxystearic acid has been studied by Basu (11).

With this problem in view the following approaches were used: (*a*) the synthesis of the analogous 12-hydroxystearic acid ester of the primary alcohol, cholesteryl carbinol, in place of the secondary alcohol, cholesterol; (*b*) separation of the desired product from the mixture resulting when the azeotropic esterification is attempted; and (*c*) the synthesis of the desired ester by the reaction of cholesteryl iodide and the silver salt of the acid.

Although the first approach mentioned does not give cholesteryl 12-hydroxystearate as originally sought, it does offer a more convenient method for the comparison of a series of hydroxystearic acid esters such as di- and trihydroxystearates. The azeotropic distillation method has been shown to be satisfactory for the preparation of hydroxystearic acid esters of primary alcohols (10, 12, 13) and its use also was found suitable in this case. The 12-hydroxystearic acid and 9,10-dihydroxystearic acid esters of cholesteryl carbinol were prepared by this method. Although it had been planned to synthesize other hydroxystearic acid esters of the carbinol, the results obtained in emulsification tests of these esters did not warrant such syntheses.

In one of the successful methods for the synthesis of cholesteryl 12-hydroxystearate, cholesterol was esterified with 12-hydroxystearic acid by the previously described azeotropic distilla-

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Previous paper in this series, THIS JOURNAL, *Pract. Pharm. Ed.*, **11**, 36(1950).
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tion method and the ester was isolated from the reaction mixture.

The second method of preparation was the reaction of cholesteryl iodide (14), with silver 12-hydroxystearate. This previously unreported salt was prepared by the method of Whitby (15) for the preparation of silver stearate.

It has been established that the catalyzed esterification of hydroxyl groups does not result in the rupture of the alkyl oxygen bond of a secondary alcohol (16, 17). Therefore, the cholesteryl 12-hydroxystearate synthesized by such a method must be of the *beta* configuration. The ester formed by the reaction of cholesteryl iodide and the silver salt is another example of a nucleophilic replacement at the three position of Δ^5-cholestene resulting in a *beta* derivative (9, 18–21).

EXPERIMENTAL[1]

3α-Carboxy-Δ^5-cholestene.—The apparatus employed was that described by Fieser (22*a*) for the preparation of the Grignard reaction under nitrogen. A Trubore stirrer shaft fitted with a Hershberg stirrer of Chromel wire was substituted for the indicated mercury seal.

To 10 Gm. of magnesium turnings previously baked under nitrogen in the above apparatus, was added rapidly, with stirring, a solution of 1 cc. of methyl iodide in 50 cc. of anhydrous ether followed by 50 Gm. of cholesteryl chloride (23) in 750 cc. of anhydrous ether. A solution of 5 cc. of methyl iodide in 100 cc. of anhydrous ether was added over a period of six hours. The reaction was refluxed and stirred during this time and for another eighteen hours. Dry carbon dioxide gas was then introduced with stirring at room temperature during a period of eight hours.

The Grignard complex was decomposed with 1 L. of a saturated solution of ammonium chloride followed by an equal volume of 20% hydrochloric acid. The resulting aqueous-ethereal suspension was extracted with 2 L. of ether and the ethereal suspension filtered to remove 8.7 Gm. of bicholesteryl. One liter of acetone was added to the filtrate, the solution concentrated to 250 cc. and placed overnight in the refrigerator. The precipitated acid was removed by filtration and recrystallized twice from xylene to yield 22.3 Gm. The acid melted at 224° to a cloudy melt, clearing sharply at 262–264°, when taken by the capillary tube method. Baker and Squire (8), who reported that the acid melted at 227–228° with preliminary softening at 224°, recorded no such cloudiness. The melting points of the methyl and ethyl esters prepared from our acid correspond to those reported by Marker, *et al.* (5, 6), and Baker and Squire (24).

Preparation of the Esters of 3α-Carboxy-Δ^5-cholestene.—(*a*) *Using Sulfuric Acid as Catalyst.*—The methyl and ethyl esters were prepared by the method of Marker, *et al.* (5, 6), and Baker and Squire (24). The *n*-propyl and *n*-butyl esters were prepared by refluxing a mixture of 5 Gm. of 3α-carboxy-Δ^5-cholestene, 80 cc. of the required alcohol, and 1 cc. of sulfuric acid for eight hours. The reaction mixture was cooled to 0° and the ester removed by filtration and washed with water. The crude ester was dissolved in 100 cc. of petroleum ether (b. p. 30–60°) and the solution extracted with 5% sodium hydroxide solution followed with water. The petroleum ether solution was dried over sodium sulfate and passed through a 2.5-cm. column of alumina (Fisher Absorption Alumina). The ester was then eluted with 180 cc. of a mixture of petroleum ether (b. p. 30–60°) and benzene (3:1) and the solvent was removed from the combined eluates by distillation under reduced pressure. The residue was dissolved in a sufficient amount of the appropriate solvent, the solution filtered to remove a small amount of insoluble material, allowed to cool, and the product removed by filtration.

(*b*) *The Azeotropic Method Using p-Toluenesulfonic Acid as Catalyst.*—A mixture of 5.0 Gm. (0.012 mole) of 3α-carboxy-Δ^5-cholestene, 0.012 mole of the alcohol, 0.15 Gm. of *p*-toluenesulfonic acid, and 100 cc. of benzene was refluxed vigorously for four hours in the apparatus described by Bell and Taub (12). The benzene was removed by distillation under reduced pressure. The crude ester, dissolved in 100 cc. of petroleum ether (b. p. 30–60°), was passed through a 2.5 x 15 cm. column of alumina and purified as described in method (*a*) above.

(*c*) *Using the Alcohol and the Acid Chloride of 3α-Carboxy-Δ^5-cholestene.*[2]—A mixture of 5.0 Gm. (0.012 mole) of 3α-carboxy-Δ^5-cholestene, 5 cc. of purified thionyl chloride (22*b*), and 50 cc. of benzene was refluxed for six hours in an apparatus protected by a calcium chloride drying tube. After the mixture had remained overnight, the solvent and excess thionyl chloride were distilled off under reduced pressure. To the crude acid chloride there was added 0.012 *M* of the appropriate alcohol and the mixture was heated for two hours on the steam bath with occasional swirling. The reaction mixture was dissolved in 100 cc. of petroleum ether (b. p. 30–60°) and the solution was passed through a 2.5 x 18 cm. column of alumina and purified as described in method (*a*) above.

The preparation of the esters of 3α-carboxy-Δ^5-cholestene is summarized in Table I.

3α-(Hydroxymethyl)-Δ^5-cholestene.[3]—(*a*) *From the Ethyl Ester of 3α-Carboxy-Δ^5-cholestene.*—The apparatus employed was the same as that used for the preparation of 3α-carboxy-Δ^5-cholestene.

A solution of 35.4 Gm. (0.08 mole) of the ethyl ester of 3α-carboxy-Δ^5-cholestene in 200 cc. of anhydrous ether was added dropwise to a well-stirred mixture consisting of 2.0 Gm. of crushed lithium aluminum hydride and 200 cc. of anhydrous ether and the mixture was stirred for one-quarter hour after all the ester solution had been added. Water was then carefully added dropwise, with stirring, until there was no further visible reaction and the mixture was poured into 500 cc. of ice-cold,

[1] The authors wish to thank Dr. Lester I. Conrad and American Cholesterol Products, Inc., for the very generous supplies of cholesterol employed in this investigation.

[2] Baker and Squire (8) reported the pure acid chloride to melt at 119–120°.

[3] While this work was in progress, the preparation of this carbinol was described by Baker and Petersen (9).

TABLE I.—ESTERS OF 3α-CARBOXY-Δ⁵-CHOLESTENE[a]

No.	Alcohol	Method (Prepn.)	Yield (%)	M. P.[b] (°C.)	Saponification No. Calcd.	Saponification No. Found
1	Methyl[c]	*a*	81	101.5–102.5	...	...
2	Ethyl[c]	*a*	81	82–3	(126.7)	(124.7)
		c	80			
3	*n*-Propyl	*a*	51	84.5–85.6	122.9	120.9
4	*n*-Butyl	*a*	53	62–3	119.2	121.7
5	*n*-Amyl	*b*	40	65–6	115.7	117.9
6	*n*-Hexyl	*b*	47	54.5–55.5	112.5	114.7
7	*n*-Heptyl	*b*	63	58–9	109.4	109.0
8	*n*-Octyl	*b*	51	44–5	106.5	109.6
9	*n*-Nonyl	*b*	60	47–8	103.8	109.1
10	*n*-Decyl	*b*	50	49–50	101.1	103.6
		c	73			
11	*n*-Undecyl	*b*	53	42–3	98.6	99.2
12	Lauryl	*c*	67	46.5–47.5	96.3	95.7
13	Myristyl	*c*	80	40.5–41.5	91.8	92.1
14	Cetyl	*c*	82	48–9	87.8	88.2
15	Stearyl	*b*	61	57–8	84.1	84.5
		c	91			

[a] Compound 1 was recrystallized from a mixture of ether and methyl alcohol; 2 from a mixture of ether and ethyl alcohol; 3, 4, and 5 from ethyl alcohol; and 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15 from acetone.
[b] On solidification from the melted state, these esters exhibit an iridescence similar to that shown by many of the cholesteryl esters (Refs. 10, 25).
[c] Compounds previously reported (Refs. 5, 6, 24).

10% sulfuric acid. The mixture was transferred to a separatory funnel, the acid layer drawn off and discarded, the ethereal layer washed with 150 cc. of 5% potassium hydroxide solution and then with water until the washings were no longer alkaline to litmus. The ethereal solution was dried with anhydrous sodium sulfate, filtered, and the solvent removed by distillation under reduced pressure. The solid residue was dissolved in 150 cc. of boiling alcohol and after cooling to room temperature the solution was kept in the refrigerator overnight. The carbinol crystallized in plates; the yield was 29.5 Gm. (92%) melting at 129.5–130.5°. A further 1.5 Gm., melting at 128–129°, was obtained by heating the mother liquor to boiling, adding hot water until a faint cloudiness appeared, and allowing the mixture to cool.

Anal.[4]—Calcd. for $C_{28}H_{46}O$: C, 83.93; H, 12.08. Found: C, 84.38; H, 11.97.

(*b*) *From 3α-Carboxy-Δ⁵-cholestene.*—In place of the condenser of the apparatus used in the above preparation of this carbinol, there was substituted a Soxhlet extractor and its condenser. The recondensed ether from a refluxing mixture of 600 cc. of anhydrous ether and 3.0 Gm. of crushed lithium aluminum hydride was used to extract 22.0 Gm. of 3α-carboxy-Δ⁵-cholestene. Refluxing and stirring were continued until one-half hour after all the acid had been extracted. Water was then added and the carbinol isolated in the same manner as previously described. The yield was 18.6 Gm. (87%) melting at 129.5–130.5°. A mixture of this carbinol with that obtained by the previous method exhibited no depression in melting point.

Preparation of the Normal Esters of 3α-(Hydroxymethyl)-Δ⁵-cholestene.—(*a*) *Acetate of 3α-(Hydroxymethyl)-Δ⁵-cholestene.*—A mixture consisting of 5.0 Gm. (0.0125 mole) of 3α-(hydroxymethyl)-Δ⁵-cholestene and 10 cc. of acetic anhydride was placed in a 50-cc. round-bottom flask fitted with a reflux condenser and a calcium chloride drying tube and refluxed for forty-five minutes. The reaction mixture was poured on ice, the solid product filtered off, thoroughly washed with water, and air-dried. The crude ester was dissolved in 50 cc. of petroleum ether (b. p. 30–60°), the solution dried over anhydrous sodium sulfate, filtered, and passed through a column of alumina (2.5 x 15 cm.). An additional 25 cc. of petroleum ether was passed through the column and elution of the ester was completed with 200 cc. of benzene. The combined petroleum ether-benzene eluate was distilled under reduced pressure and the residue was recrystallized from 50 cc. of alcohol. The acetate crystallized in plates; the yield was 4.6 Gm. (83%), melting at 82–83°.

(*b*) *General Method.*—All the normal esters except the acetate were prepared by reaction of the carbinol with the appropriate acid chloride by method (*c*) described above for the preparation of the ester of 3α-carboxy-Δ⁵-cholestene. The acid chlorides were prepared by the method of Bauer (26).

Hydroxystearic Acid Esters of 3α-(Hydroxymethyl)-Δ⁵-cholestene.—A mixture of 4.0 Gm. (0.01 mole) of the carbinol, 0.012 mole of the acid, 0.15 Gm. of *p*-toluenesulfonic acid, and 100 cc. of benzene was refluxed vigorously for six hours in the azeotropic apparatus of Bell and Taub (12). After reaching room temperature the reaction mixture was filtered to remove any unreacted acid and the residue washed with benzene. The combined filtrates were passed through a 2.5 x 15 cm. column of alumina and the ester eluted with 300 cc. of benzene. After removing the benzene from the combined eluates by distillation under reduced pressure, the esters were recrystallized from alcohol.

The preparation of the esters of 3α-(hydroxymethyl)-Δ⁵-cholestene is summarized in Table II.

Cholesteryl 12-Hydroxystearate.—*Prepared by the Azeotropic Method.*—A mixture of 19.4 Gm. (0.05 mole) of cholesterol, 15.0 Gm. (0.05 mole) of 12-hydroxystearic acid, 0.6 Gm. of *p*-toluenesulfonic

[4] Analysis by Clark Microanalytical Laboratory, Urbana, Ill.

TABLE II.—ESTERS OF 3α-(HYDROXYMETHYL)-Δ⁵-CHOLESTENE[a]

No.	Acid	Yield (%)	M. P. (°C.)	Saponification No. Calcd.	Saponification No. Found
1	Acetic	83	82–3	126.7	123.9
2	Butyric	83	66–7	119.2	117.4
3	Caproic	76	45–6	112.5	112.5
4	Caprylic	74	49–50	106.5	109.3
5	Capric	76	51.5–52	101.1	97.6
6	Lauric	86	44–5	96.3	94.2
7	Myristic	85	41.5–43	91.8	88.3
8	Palmitic	77	48.5–49.5	87.7	82.4
9	Stearic	83	56–7	84.1	85.0
10	12-Hydroxystearic	70	60–1	81.9	84.1
11	9,10-Dihydroxystearic	56	109–10	80.1	81.8

[a] Compounds 1, 2, 10, and 11 were recrystallized from alcohol (95%); compounds 3, 4, 5, 6, 7, 8, and 9 from absolute alcohol.

acid, and 200 cc. of benzene was refluxed vigorously for six hours in the azeotropic apparatus of Bell and Taub (12). After reaching room temperature the reaction mixture was filtered and the benzene was removed from the filtrate by distillation under reduced pressure. The residue was taken up in 800 cc. of ether and extracted with 100-cc. portions of a solution of 5% sodium hydroxide in a 25% alcohol-water mixture until the ethereal layer was free of any precipitate. After the ethereal layer was washed free of base and dried over sodium sulfate, the solvent was removed by distillation. The crude ester was dissolved in 400 cc. of petroleum ether (b. p. 30–60°), passed through a 2.5 x 25 cm. column of alumina, and eluted with a mixture of petroleum ether (b. p. 30–60°) and benzene (3:1). The solvents were removed by distillation under reduced pressure and the residue recrystallized a total of five times, alternating first from acetone and then petroleum ether (b. p. 30–60°). The yield was 5.8 Gm. melting at 72–75°. When a portion was melted, allowed to solidify, and then powdered, the melting point was sharp at 78–79°. However, recrystallizing such preheated material returned the melting point to 72–74°.

Anal.—Calcd. for $C_{45}H_{80}O_2$: ester value, 83.6; OH, 2.53. Found: ester value, 84.0; OH, 2.52.

Silver 12-Hydroxystearate.—To a solution of 4 Gm. (0.013 mole) of 12-hydroxystearic acid dissolved in 125 cc. of alcohol at 50° were added 90 cc. of 28% ammonium hydroxide and 2.26 Gm. (0.013 mole) of silver nitrate dissolved in 5 cc. of warm water. The silver salt was allowed to precipitate for three hours while the solution was kept at 50°, filtered, washed with alcohol, and allowed to dry on a porous plate. The yield was 3.1 Gm. melting at 208–209°.

Cholesteryl 12-Hydroxystearate.—*Alternate Preparation.*—A mixture of 1.8 Gm. of powdered silver 12-hydroxystearate, 2.1 Gm. of cholesteryl iodide (14), and 25 cc. of benzene was refluxed for three hours. The reaction mixture was filtered, the residue washed with ether, and the solvents removed from the combined filtrates by distillation under reduced pressure. The semisolid material remaining was extracted with four 25-cc. portions of acetone. The combined acetone extracts were refluxed with Norite®, filtered, and the acetone removed by distillation under reduced pressure. The crude ester was extracted with 100 cc. of petroleum ether (b. p. 30–60°), filtered, and passed through a 2.5 x 12 cm. column of alumina. The column was washed with 200 cc. of benzene to remove a small amount of material which fluoresced under ultraviolet light. The ester was then eluted from the column with 200 cc. of commercial ether. The ether was removed from the eluate by distillation and the ester recrystallized twice from acetone. The yield was 0.3 Gm. of material melting at 72–75° which, when preheated, melted at 78–79°. There was no depression in melting point when a sample was mixed with cholesteryl 12-hydroxystearate prepared by the azeotropic method.

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Water-in-Oil Emulsifying Agents*

VII. An Improved Method of Evaluating Emulsifying Efficiencies

By LEE WORRELL† and JOSEPH E. SINSHEIMER‡

The ability of normal esters of 3α-carboxy-Δ⁵-cholestene, normal esters of 3α-(hydroxymethyl)-Δ⁵-cholestene and steroid hydroxystearic acid esters to potentiate the emulsifying efficiency of cholesterol in petrolatum has been studied. A comparison of the emulsifying ability of some derived steroids to that of cholesterol has been made. The nature of the quantal response occurring in connection with the procedure used for these studies has been recognized and the Litchfield-Wilcoxon method for the statistical evaluation of such responses has been applied.

In the previous paper of this series (1) the synthesis of the following series of esters has been reported: (*a*) esters of 3α-carboxy-Δ⁵-cholestene, (*b*) esters of 3α-(hydroxymethyl)-Δ⁵-cholestene, and (*c*) hydroxystearic acid esters. The abilities of these esters when mixed with cholesterol to increase the amount of water emulsified in petrolatum by cholesterol alone have been evaluated and the results are reported in this paper. A quantitative comparison of these abilities with similar abilities previously shown by esters of cholesterol should allow further insight into the relationship of structure and of molecular weight to potentiation of emulsifying power.

The method of evaluation used was a modification of the Powers-Cataline method presented previously in this series of papers (2, 3), and is described in detail below. Two significant changes were made. The amount of 3%-cholesterol-and-3%-ester-in-petrolatum base was reduced from 5 Gm. to 2 Gm. per trial. Since the *percentage* of water, as well as the absolute *amount* of water, emulsified changed as the amount of base was changed, as is shown in Table I, the term "Emulsifying Efficiency Per Cent" was dropped as misleading and results are reported as amount of water emulsified under standard conditions.

The second change was in the method of reading the results. Both the evaluation procedure of Powers and that of Cataline note that a "decision" was reached as to the maximum amount of water emulsified. In the case of the former this decision was made on the basis of one series while in the latter procedure at least two series were run before the decision was reached. In both procedures these series were carried only as far as the first negative results. It was hoped that a more precise decision could be reached as to the maximum level of emulsification by defining the maximum level as the amount of water that still results in complete emulsification in four out of five tests. To arrive at this maximum level experimentally, a process of bracketing the maximum value and reducing the size of this bracket was used.

The cholesterol was taken from a single sample of 200 Gm. (m. p. 148.5–149.5°) (Eastman), while the source of the petrolatum was a 10-lb. container of petrolatum, snow white, U. S. P. alba (Standard Oil).

EXPERIMENTAL

Method.—The apparatus was that described by Cataline with substitution of a stainless-steel stirrer for the glass stirrer. Rotation was maintained at 2200 r. p. m. as previously described.

The components of the mixture to be tested, each in the amount equal to 3% of the total weight of the base, were dissolved with stirring in petrolatum by heating on an oil bath at 125–135° for ten minutes. The mixture was allowed to cool with frequent stirring until solidified. This base was remelted as required in an oil bath of 80–90° and the same standard quantity was poured and weighed into a series of tared eight-inch test tubes. The required amount of distilled water was added by graduated pipet and the test tube was placed for a minimum of five minutes in a water bath at 85 ± 2°.

The heated test tube was clamped into place with the bearing of the stainless-steel stirrer resting on the bottom of the test tube. The motor was started and the clamps holding the tube released sufficiently to permit the tube to be raised and lowered. Uniform mixing over the length of the tube was insured in this manner. At the time of the signs of definite emulsification an interval timer set for one-half minute was released. At the end of this period the motor was turned off long enough to rotate and move the tube up and down by hand in order to mix any water trapped at the base of the tube. The

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TABLE I.—PRELIMINARY EMULSIFYING EFFICIENCIES

Series No.	System Evaluated	Base (Gm.)	Efficiency (cc.)	Efficiency (%)[a]
1	Cholesterol	1	3	300
2	Cholesterol	2	7–8	350–400
3	Cholesterol	3	13.5	450
4	Cholesterol	4	20	500
5	Cholesterol	5	25	500
6	Cholesterol	6	33	550
7	Cholesterol-cholesteryl-stearate	4	>32	>800[b]
8	Cholesterol-cholesteryl-stearate	2	16	800
9	Cholesterol-cholesteryl-laurate	4	>32	>800[b]
10	Cholesterol-cholesteryl-laurate	2	13	650
13	Petrolatum, *per se*	2	<1	0
14	Petrolatum, *per se*	5	<1	0

[a] Per cent efficiency is the ratio of cubic centimeters of water emulsified to the grams of base used.

[b] The next level was beyond the capacity of the tube.

timer was reset for one-half minute, stirring restarted, and at the end of this time the tube was slowly lowered from the stirrer with the stirrer still rotating at 2200 r. p. m. Just as the stirrer was being withdrawn from the top of the emulsion, the motor was turned off.

The decision was made as to whether or not complete emulsification had occurred on the basis of whether or not water could be detected and whether or not the emulsion had rotated freely in contact with the test tube. Free rotation and the accompanying appearance of air pockets and folds were taken as signs of incomplete emulsification. In the presence of any doubt at this point, stirring was started once again and when maximum speed was obtained the withdrawal of the test tube was repeated until a more definite decision could be reached.

Tests were continued in this manner upon a series of samples of a given base, at all times attempting the greatest duplication of experimental conditions. The level of maximum emulsification was sought by first bracketing this level with two positive results and two negative results and then extending the positive results to at least four out of five positive results at as high a level as possible.

Application.—Since we wished to compare not only the emulsifying capacity of the cholesterol-new-ester systems to that of cholesterol alone, but also to that of cholesterol-previously-reported-ester systems, and since the maximum levels of emulsification, as newly defined and determined, differed significantly from those previously reported because of changes in the procedure, new levels were determined for cholesterol-cholesteryl-stearate and for cholesterol-cholesteryl-laurate, as well as for cholesterol alone. These values may be used for comparative purposes and appear as series numbers 2, 8, and 10 in Table I.

Maximum levels of emulsification were then determined for systems composed of cholesterol and each of the newly synthesized compounds using the method described above and 2-Gm. quantities of base. Tables II and III summarize most of these results. In Table IV are summarized the maximum levels of water emulsified by the various steroids in petrolatum for comparison with the emulsifying ability of cholesterol in petrolatum. The results of the evaluation of 3% mixtures of three representative esters are also included.

TABLE II.—MAXIMUM AMOUNT OF WATER EMULSIFIED BY TWO-GRAM SAMPLES OF MIXTURES OF CHOLESTEROL (3%), ESTER OF 3α-(HYDROXYMETHYL)-Δ⁵-CHOLESTENE (3%) AND PETROLATUM

Series No.	Ester	Water (cc.)
17	Stearate	17
18	Palmitate	15
19	Myristate	14
20	Laurate	14
21	Caprate	14
22	Caprylate	13
23	Caproate	11
24	Butyrate	15
25	Acetate	11
26	12-Hydroxystearate	13
27	9,10-Dihydroxystearate	13

TABLE III.—MAXIMUM AMOUNT OF WATER EMULSIFIED BY TWO-GRAM SAMPLES OF MIXTURES OF CHOLESTEROL (3%), ESTER OF 3α-CARBOXY-Δ⁵-CHOLESTENE (3%) AND PETROLATUM

Series No.	Ester	Water (cc.)
28	Stearyl	15
29	Cetyl	15
30	Myristyl	15
31	Lauryl	12
32	*n*-Undecyl	10
33	*n*-Decyl	11
34	*n*-Nonyl	11
35	*n*-Octyl	12
36	*n*-Heptyl	10
37	*n*-Hexyl	11
38	*n*-Amyl	12
39	*n*-Butyl	12
40	*n*-Propyl	11
41	Ethyl	11
42	Methyl	10

In addition to the emulsifying efficiencies of the systems summarized in Tables II and III, the potentiating effect of a 2-Gm. sample of cholesteryl 12-hydroxystearate in a 3% ester, 3% cholesterol and petrolatum mixture was also determined as

series No. 49. The maximum amount of water emulsified was found to be 15 cc.

It was found that the results of the evaluations thus far reported were not altered after six months of shelf storage. Therefore, it was decided in future testing to make an immediate decision as to whether emulsification had occurred and to eliminate the shelf-storage period.

In reviewing the data reported in Tables II to IV, the two most disturbing facts were: (*a*) the high value in relation to its place in the series observed for the butyric acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene (series No. 24) and (*b*) the lack of uniform increase in emulsifying ability with increase in molecular weight for the series of esters of 3α-carboxy-Δ⁵-cholestene. Although general qualitative conclusions could be drawn from these results, no quantitative comparison could be made. There were left unanswered the following questions:

TABLE IV.—MAXIMUM AMOUNT OF WATER EMULSIFIED BY TWO-GRAM SAMPLES OF MIXTURES OF VARIOUS STEROIDS (3%) IN PETROLATUM

Series No.	Steroid	Water (cc.)
43	3α-(Hydroxymethyl)-Δ⁵-cholestene	3
44	3α-Carboxy-Δ⁵-cholestene[a]	<1
45	Epicholesterol	1
2	Cholesterol	7–8
46	Cholesteryl 12-hydroxystearate	<1
47	Stearyl ester of 3α-carboxy-Δ⁵-cholestene	<1
48	Stearic acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene	<1

[a] This acid is insoluble in petrolatum and was tested as suspension (3).

What were the limits of error with any given determination? In comparing any two values were these limits being exceeded?

The need for a statistical evaluation of the results obtained or for a method that would permit such an evaluation was recognized.

A large number of tests had been completed by the method of bracketing in order to obtain the data reported. During the use of this bracketing process, examples of positive emulsification far in excess of the final maximum level of emulsification were discovered as well as negative emulsification at levels below that of the final maximum level. In order to investigate the frequency of such positive and negative results, it was decided to investigate the 3%-cholesterol-in-petrolatum system more extensively. Accordingly, five sets of increasing amounts of water added to 2-Gm. quantities of this base were examined for successful emulsification by the same method used to obtain the data of Tables I to IV.

The bracketing procedure of this method, of course, was deleted. Instead, all the members of a given set were placed in the water bath at the same time and after a minimum of five minutes a tube was withdrawn at random and evaluated for emulsification.

The range of the amounts of water was chosen with the intention of obtaining all positive results at the lower amounts and all negative results at the higher amounts.

Table V is a summary of the results obtained for the five sets of cholesterol so investigated.

Table V confirmed the opinion gained during the application of the bracketing method, that in this type of emulsification procedure the values obtained for the maximum level of emulsification may vary from trial to trial. If the maximum level had been determined with just a single set, levels of 9, 7, or 8 cc. could have been reported. The danger of inconsistent results even occurs when the criterion of four out of five positive results is used. In addition, there is the occurrence of positive results beyond the first negative result to consider. The problem, therefore, became what criterion should be set which would have a high degree of reproducibility and still be experimentally possible. Furthermore, a method was needed to estimate the degree of reproducibility of such a criterion.

The Quantal Response.—Since the emulsification testing procedure used had as its basis the decision of whether emulsification had occurred or had not occurred, it is essentially an "all-or-none effect" or quantal response rather than a graded response. It was realized that instances of all-or-none effects are to be found in connection with biological problems and that their statistical evaluation was important (4).

Munch and Garlough (5) credit Shackell and coworkers (6–8) in 1923–1925 and Trevan (9) in 1927 with the first recognition that the relationship between dose and effect follows a sigmoid curve. Trevan established these sigmoid curves to be asymmetric in character with less change in percentage effect with the same increment in the case of the larger doses than with the smaller doses. He further showed that at least twenty to thirty animals should be tested at each level when determining these curves. Trevan was also among the first to suggest that the level at which 50% of the tested animals gave positive responses or ED_{50} be used as

TABLE V.—EMULSIFICATION OF TWO-GRAM SAMPLES OF CHOLESTEROL (3%) IN PETROLATUM

	Volume of Water (cc.)										
Set No.	3	4	5	6	7	8	9	10	11	12	13
1	+[a]	+	+	+	+	+	+	−[b]	−	−	−
2	+	+	+	+	+	−	−	+	+	+	−
3	+	+	+	+	+	+	+	−	−	−	−
4	+	+	+	+	+	+	+	−	−	+	−
5	+	+	+	+	+	+	−	+	+	−	−
Per Cent +	100	100	100	100	100	80	60	40	40	40	0

[a] + = complete emulsification.
[b] − = incomplete emulsification.

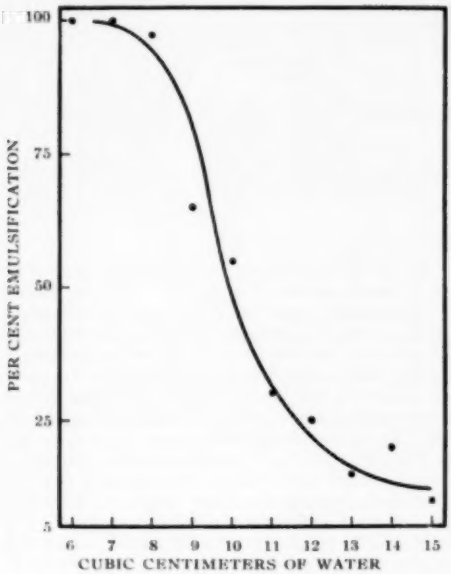

Fig. 1.—Percentage emulsification curve for the cholesterol system.

the most characteristic and best defined level of a given drug and its response.

To establish the nature of the quantal responses being studied in connection with the emulsification procedure used in this investigation, the procedure need only be modified in order to follow the criteria of Trevan by increasing the number of sets of determinations to twenty. This was then done for both cholesterol-(3%)-in-petrolatum system and the cholesterol-(3%)-and-cholesteryl-stearate-(3%)-in-petrolatum system. In this manner it was hoped to study both the primary emulsifier, cholesterol, and a system containing a typical potentiating ester. A summary of these tests is given in Table VI while possible curves fitted to these data are presented in Figs. 1 and 2.

An inspection of these curves indicates their sigmoid shape and the possibility of asymmetry. This asymmetry again appears to be characterized by less change in percentage effect with the same increment in the case of the larger water levels than with the smaller levels.

A normal distribution curve can be reduced to a straight line plot by the use of arithmetic-probability paper. However, it is the general pharmacological practice in dealing with asymmetric dose-response curves to plot the logarithm of the dose on probability paper in order to approach more satisfactorily a straight line. The results of making similar logarithmic-probability graphs for water-level-emulsification distributions are shown in Fig. 3 for the cholesterol system and in Fig. 4 for the cholesterol-cholesteryl-stearate system. The data reported in Table VI were again used for these plots.

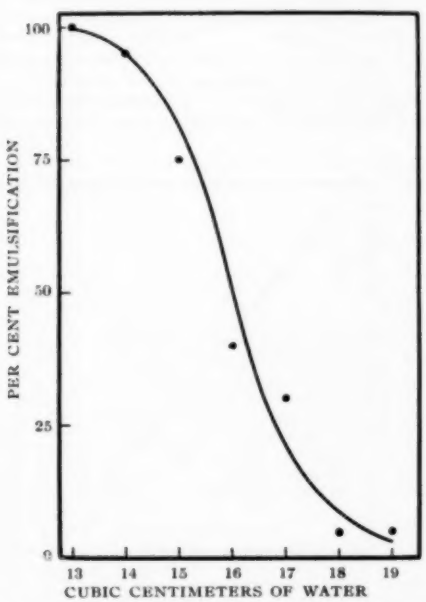

Fig. 2.—Percentage emulsification curve for the cholesterol-cholesteryl-stearate system.

TABLE VI.—PERCENTAGE EMULSIFICATION WITH INCREASING AMOUNTS OF WATER FOR THE CHOLESTEROL AND CHOLESTEROL-CHOLESTERYL-STEARATE SYSTEMS

Amount of Water (cc.)	Per Cent Emulsified*	
	Cholesterol	Cholesterol-Cnolesteryl-Stearate
6	100	..
7	100	..
8	95	..
9	65	..
10	55	..
11	30	..
12	25	..
13	15	100
14	20	95
15	10	75
16	..	40
17	..	30
18	..	5
19	..	5

* Per cent emulsified is based upon the ratio of the number of tests resulting in complete emulsification to the total of twenty tests at a given amount of water.

Table VI and Figs. 1–4 demonstrate the similarity of the all-or-none effect as found in the emulsification testing procedure used and that of the same effect previously established and well confirmed in regard to the action of drugs and their responses. The latter effect has been the basis of extensive work in the quantitative comparison of drug potency, toxicity, and effects. It was believed that the statistical methods developed to obtain such quantitative comparisons would be of aid in the statistically similar problem found in connection with the method of emulsification used.

TABLE VII.—MEDIAN EMULSION LEVELS AND SLOPES OF CHOLESTEROL-(3%)-ESTER-(3%)-PETROLATUM SYSTEMS

Series No.	Ester	Median Emulsion Level (cc.)	Slope
49	Cholesteryl stearate	16.0 (15.5–16.5)	1.08 (1.06–1.10)
50	Stearic acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene	16.3 (15.7–17.0)	1.08 (1.04–1.12)
51	Stearyl ester of 3α-(carboxy)-Δ⁵-cholestene	15.8 (14.9–16.7)	1.09 (1.04–1.14)
52	Cholesteryl 12-hydroxystearate	14.0 (12.8–15.2)	1.13 (1.05–1.21)
53	Acetic acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene	14.4 (13.4–15.4)	1.12 (1.06–1.19)
54	Ethyl ester of 3α-carboxy-Δ⁵-cholestene	13.5 (12.5–14.6)	1.13 (1.07–1.20)

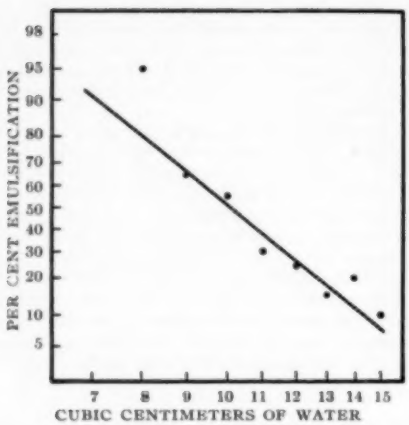

Fig. 3.—Logarithmic probability curve for the cholesterol system.

The method of Litchfield and Wilcoxon (10) appeared to be well adapted to our needs as it is extensive enough to give from a small number of sets of data: (*a*) an evaluation with the limits of error of the level at which 50% emulsification occurs and (*b*) a method of comparing those 50% levels and the limits of error of such comparisons.

With this method it is possible to report results at confidence limits for any probability. In our work all results are reported at confidence limits for 19/20 probability, the probability for which the method was originally developed.

The nomenclature of Litchfield and Wilcoxon has been modified to conform to our specialized requirements. Median Emulsion Level (EL_{50}) is used in place of the standard, Median Effective Dose (ED_{50}) and Emulsion Ratio (E. R.) is substituted for Potency Ratio (P. R.).

The Application of a Statistical Evaluation.—The first data obtained which could be statistically evaluated were those presented in Table VI. The following values were obtained: cholesterol, EL_{50} = 10.2 (9.6 to 10.9); cholesterol-cholesteryl-stearate, EL_{50} = 16.0 (15.5 to 16.5); E. R. = EL_{50} cholesterol-cholesteryl-stearate/EL_{50} cholesterol = 1.57 (1.47 to 1.70).

When the same calculations were made on the basis of only the first five of the twenty sets determined, the following results were obtained: cholesterol, EL_{50} = 10.3 (9.0 to 11.7); cholesterol-cholesteryl-stearate, EL_{50} = 16.1 (14.9 to 17.4); E. R. = 1.56 (1.35 to 1.79).

On the basis of these results, it was decided to make future statistical evaluations with the limited experimental results from five sets of determinations. It was realized, of course, that the more sets determined, the narrower would be the confidence limits. The use of five sets, however, appeared to be consistent with the limitations of the availability of material and time, while still yielding results with satisfactory confidence limits.

The Litchfield and Wilcoxon method permits a calculation of the slopes of the curves of the systems to be compared. These slopes are then used to test the parallelism of the curves. Only in the absence of a significant deviation in the parallelism of the two systems can a general comparison be made. If a significant deviation in parallelism is found, then the comparison is limited in this method to the EL_{50} values.

In the comparison of the cholesterol and cholesterol-cholesteryl-stearate systems significant deviations in parallelism were found both on the basis of five sets of determinations and the twenty sets. Therefore, in case of data based upon twenty sets, the Emulsion Ratio (E. R.), for the comparison of the cholesterol and cholesterol-cholesteryl-stearate systems, means that the latter system will emulsify 1.57 (1.47 to 1.70) times as much water at the EL_{50} level as the former.

In an attempt to answer the various questions left unanswered following the determination of emulsifying efficiencies by the bracketing method, selected steroid-in-petrolatum systems were experi-

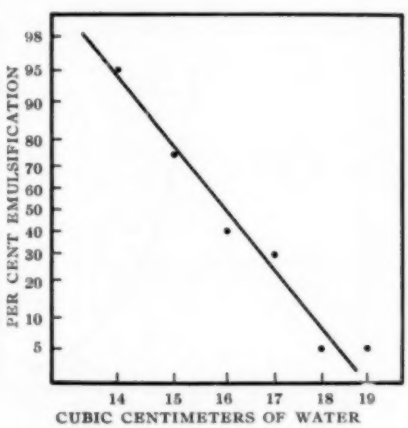

Fig. 4.—Logarithmic probability curve for the cholesterol-cholesteryl-stearate system.

TABLE VIII.—MEDIAN EMULSION LEVELS AND SLOPES OF STEROID-(3%)-PETROLATUM SYSTEMS

Series No.	Steroid	Median Emulsion Level (cc.)	Slope
55	Cholesterol	10.2 (9.6–10.9)	1.31 (1.22–1.40)
56	Epicholesterol	1.62 (0.92–2.84)	2.46 (0.82–7.38)
57	3α-Carboxy-Δ⁵-cholestene	[a]	...
58	3α-(Hydroxymethyl)-Δ⁵-cholestene	7.1 (6.4–7.9)	1.12 (1.06–1.19)
59	Cholesteryl 12-hydroxystearate	[a]	...
60	Stearic acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene	[a]	...
61	Stearyl ester of 3α-carboxy-Δ⁵-cholestene	[a]	...
62	Petrolatum, *per se*	[a]	...

[a] At both the 1- and 2-cc. levels all tests failed to emulsify.

mentally re-examined on the basis of five sets of determinations and then the results were evaluated statistically.

These values together with those already determined upon the basis of twenty sets, the cholesterol and cholesterol-cholesteryl-stearate systems, are recorded in Tables VII and VIII. In Table VII are summarized the EL_{50} and slopes of those systems containing cholesterol (3%) and an ester (3%) while in Table VIII are summarized the EL_{50} and slopes of systems of a single steroid (3%) in petrolatum. In addition, there are recorded in Table IX the ratios of various pairs of these median emulsion levels, i. e., the emulsion ratios.

DISCUSSION

The Method of Evaluation.—In comparing the action of drugs it has been established and fully accepted that the use of the median effective dose (ED_{50}) is the most satisfactory means of making the comparison (4, 5). The statistical error of estimation tends to be a minimum at this level. Thus, in the treatment of the quantal response found in this investigation a similar median emulsion level has been adopted. Not only is the statistical comparison at this level more accurate than at other levels, but also it is felt to be superior to any "experimental estimation" of a given level. It is certainly superior to any such experimental estimation so far attempted.

The previous work of Powers (2), of Cataline (3), and the preliminary work of this investigation were examples of such experimental estimations. The work of Powers was the experimental estimation of the 100% emulsification level, based upon one set. Cataline's method was also an estimation of the 100% emulsification level, using two sets. The method of bracketing developed in the first part of this paper was an experimental estimation of the 80% emulsification level in which the number of necessary tests varies with each determination.

The Slopes of the Water-Level-Emulsification Curves.—In the process of determining the median emulsion levels and emulsion ratios by the method of Litchfield and Wilcoxon (10), the slopes of water-level-emulsification curves can be calculated. These slopes have been recorded in Tables VII and VIII. With the exception of the values obtained for the epicholesterol and cholesterol-in-petrolatum systems, these slopes indicate no significant deviation in parallelism for these curves. Although the exact physical meaning of the difference exhibited by the epicholesterol and cholesterol-in-petrolatum systems is not understood, it is well to point out the existence of this difference. Because of the difference in slope, emulsion ratios involving these systems are limited to a comparison of median emulsion levels only. Also accompanying the difference in slope is the inference that the mode of action of the epicholesterol and cholesterol-in-petrolatum systems is different than that of the other systems investigated.

Ester Potentiation.—The results obtained by the bracketing method are certainly not invalid, al-

TABLE IX.—EMULSION RATIOS

Series No.	The Ratio of System *A*	to System *B*	Emulsion Ratios (E. R.)
1	Cholesteryl stearate	Cholesterol	1.57 (1.47–1.70)
2	Cholesteryl carbinyl stearate	Cholesterol	1.60 (1.48–1.73)
3	Stearyl cholesteryl-carboxylate	Cholesterol	1.55 (1.42–1.69)
4	Cholesteryl carbinyl acetate	Cholesterol	1.41 (1.29–1.54)
5	Ethyl cholesteryl-carboxylate	Cholesterol	1.32 (1.19–1.47)
6	Stearyl cholesteryl-carboxylate	Ethyl cholesteryl-carboxylate	1.17 (1.06–1.29)
7	Cholesteryl carbinyl stearate	Cholesteryl carbinyl acetate	1.13 (1.04–1.23)
8	Cholesteryl carbinyl acetate	Ethyl cholesteryl-carboxylate	Not significant
9	Cholesteryl carbinyl stearate	Stearyl cholesteryl-carboxylate	Not significant
10	Cholesteryl carbinyl stearate	Cholesteryl stearate	Not significant
11	Cholesteryl stearate	Stearyl cholesteryl-carboxylate	Not significant
12	Cholesteryl stearate	Cholesteryl 12-hydroxystearate	1.14 (1.06–1.22)
13	Cholesterol	Epicholesterol	6.30 (3.64–10.9)
14	Cholesterol	Cholesteryl carbinol	1.44 (1.28–1.61)

Notes: (*a*) Cholesteryl carbinol has been used as a synonym for 3α-(hydroxymethyl)-Δ⁵-cholestene and cholesteryl carboxylic acid for 3α-carboxy-Δ⁵-cholestene.
(*b*) All systems named as esters have the general formula: ester (3%), cholesterol (3%), and petrolatum (94%), while all systems named as sterols have the general formula: sterol (3%) and petrolatum (97%).

though as was previously pointed out they are limited in their usefulness. When supplemented by the median emulsion level results reported in Tables VII and VIII, and their emulsion ratios reported in Table IX, the conclusion was reached that the potentiation by the esters of 3α-carboxy-Δ⁵-cholestene and by the esters of 3α-(hydroxymethyl)-Δ⁵-cholestene showed a similar pattern of increase in potentiation to that already described for esters of cholesterol. This conclusion is based upon: (1) the gradual, but not entirely consistent increase in maximum levels of emulsification obtained by the bracketing method; (2) the significant differences in the increased emulsifying efficiencies of the systems containing the stearyl ester of 3α-carboxy-Δ⁵-cholestene as compared to the ethyl ester of the same acid and of both these ester systems as compared to the cholesterol-in-petrolatum systems; (3) the similar significant differences recorded for the systems containing the stearic acid and acetic acid esters of 3α-(hydroxymethyl)-Δ⁵-cholestene; (4) the absence of any significant differences in the emulsifying efficiencies of systems containing the stearyl ester of 3α-carboxy-Δ⁵-cholestene, stearate ester of 3α-(hydroxymethyl)-Δ⁵-cholestene or cholesteryl stearate; and (5) the similar absence of any significant difference in the emulsifying efficiency of the system containing the ethyl ester of 3α-carboxy-Δ⁵-cholestene compared to the efficiency of the system containing the acetate ester of 3α-(hydroxymethyl)-Δ⁵-cholestene.

The general conclusions of the similarity of the potentiation of the emulsifying ability of cholesterol by the two new series of esters and by the previously investigated cholesteryl esters, and of an increase in potentiation accompanying an increase in the molecular weight of the esters, were made. However, it becomes evident that it is difficult to show an increase in potentiation accompanying the increase in molecular weight of very closely related esters. No attempt has been made, for example, to show a difference in potentiation caused by the methyl and ethyl esters of 3α-carboxy-Δ⁵-cholestene. In order to show such a difference in potentiation, using the method of high-speed stirring as developed to this point, a very extensive number of individual emulsion tests would be required. Only in this manner would it be possible to approach median emulsion levels with narrow enough confidence limits to permit the calculation of significant emulsion ratios.

Potentiation by Hydroxystearate Esters.—It was concluded, contrary to previously reported indications (3), that hydroxystearate esters did not show a potentiation in the emulsifying ability of cholesterol greater than that of their corresponding stearate esters. Upon the basis of the bracketing method of evaluation, the 12-hydroxystearic acid and 9,10-dihydroxystearic acid esters of 3α-(hydroxymethyl)-Δ⁵-cholestene were less efficient than the stearic acid ester of the same carbinol. Using the same method of evaluation, cholesteryl 12-hydroxystearate was also less efficient than cholesteryl stearate. This decrease in emulsifying efficiency was found to be statistically significant when the median emulsion level of cholesteryl stearate was compared to that of cholesteryl 12-hydroxystearate.

Steroid-(3%)-Petrolatum Systems.—The inability of steroid esters to serve as emulsifying agents was reconfirmed. The results of the attempts to determine median emulsion levels for selected steroid esters are summarized in Table X. There was no test resulting in emulsification at either the 1- or 2-cc. levels of the systems containing 3% of the stearic acid ester of 3α-(hydroxymethyl)-Δ⁵-cholestene, stearyl ester of 3α-carboxy-Δ⁵-cholestene, or cholesteryl 12-hydroxystearate. In addition, when petrolatum *per se* was evaluated, there was a similar absence of emulsification. These results confirm the results previously obtained by the bracketing method and are in agreement with those reported for esters of cholesterol (3).

There are also summarized in Table VIII the median emulsion levels obtained for the nonester steroids synthesized during this investigation. In Table IX are listed the emulsion ratios for these compounds. They appear to follow the criteria of Janistyn (11) that a steroid must possess solubility in the organic phase and availability of the polar group to be a good emulsifying agent. Langmuir, Schaefer, and Sobotka (12), employing monolayer films of sterols, have demonstrated episterols to be hydrophobic and the normal sterols to be hydrophilic.

The availability of the hydroxyl group is postulated as an explanation for these differences.

The median emulsion levels found for 3α-(hydroxymethyl)-Δ⁵-cholestene, cholesterol, and epicholesterol are constant with such conclusions. The poor emulsifying ability of epicholesterol could thus be explained on the basis of the lack of availability of the hydroxyl group. In addition, the intermediate emulsifying ability, between that of epicholesterol and cholesterol, determined for 3α-(hydroxymethyl)-Δ⁵-cholestene could be explained in a like manner. Since this carbinol is of the *alpha* series, the same as epicholesterol, the lack of availability of the hydroxyl group must again be a factor in its poorer emulsifying ability than that of cholesterol. The fact that it possesses an ability in excess of that of epicholesterol could be caused by the greater availability of the hydroxyl group due to the latter's increased distance from the rest of the molecule.

The acid steroid, 3α-carboxy-Δ⁵-cholestene, is not only of the *alpha* series but is also insoluble in petrolatum. This would explain its inability to serve as an emulsifying agent.

SUMMARY AND CONCLUSIONS

1. The ability of fifteen normal esters of 3α-carboxy-Δ⁵-cholestene to potentiate the emulsifying efficiency of cholesterol in petrolatum was studied and found to be similar to that of the cholesteryl esters.

2. The ability of nine normal esters of 3α-(hydroxymethyl)-Δ⁵-cholestene to potentiate the emulsifying efficiency of cholesterol in petrolatum was studied and found to be similar to that of the cholesteryl esters.

3. Three steroid hydroxystearates have been found to be less efficient than their corresponding stearate esters in the ability to potentiate the

emulsifying efficiency of cholesterol in petrolatum.

4. A comparison of the emulsifying ability of cholesterol, epicholesterol, 3α-(hydroxymethyl)-Δ^5-cholestene, and 3α-carboxy-Δ^5-cholestene in petrolatum has been made.

5. Recognition has been made of the nature of the quantal response occurring in the application of the Powers-Cataline method of comparing emulsifying systems.

6. The Powers-Cataline method of comparing emulsifying systems has been expanded and modified to permit a statistical evaluation of the results.

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The Effect of Age and of Heat on the "Ferment" in *Cascara sagrada**

By M. CHRISTINE GOSSELIN and CHARLES W. BAUER†

The hydrolytic agent in *Cascara sagrada* has been isolated and a method of analysis for the hydrolytic agent in different lots of *Cascara sagrada* has been worked out and evaluated. The agent has been isolated as an impure residue which is a black, shiny, glass-like, sticky substance with a melting point of about 220°.

IN 1888 Meier and Webber (1) reported that freshly harvested *Cascara sagrada* contained a "ferment" that produced a "griping effect" when this drug was used by human beings as a laxative. This griping effect was not produced by the aged *Cascara sagrada* which "did not contain the ferment." These workers did not identify the ferment, nor did they give the age or sex of any of the experimental subjects. Furthermore they failed clearly to outline their experimental procedure for evaluation by future investigators.

In 1904 Jowett (2) identified the ferment as emulsin since he found that it would readily hydrolyze amygdalin. He made a concentrated extract of this ferment and found that it did not produce a griping effect when it was administered to dogs and to human beings. He did not explain how he determined the absence of a griping effect.

The work of Meier and Webber was responsible for the statement in the monograph under "Cascara Sagrada" in the *United States Pharmacopeia:* "Cascara Sagrada should be collected at least one year before being used for medicinal preparations."

It is well known that the fresh bark gives a troublesome precipitate, that is not given by the aged bark, in the making of the official Cascara Sagrada Fluidextract.

It is a general assumption that the reason for the aging of *Cascara sagrada* before being used for medicinal preparations is not to avoid a troublesome precipitate in making the fluidextract, but to prevent the griping effect of the ferment. This assumption is based on a proposal made in 1950 to the members of the United States Pharmacopoeial Revision Committee

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This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by M. Christine Gosselin in partial fulfillment of the requirements of Master of Science in Pharmacy.
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that *Cascara sagrada* be heated at 100° for one hour in order to destroy the ferment and thus avoid the necessity of aging.

This proposal met with vigorous opposition because it was not supported by experimental evidence that heat would destroy the ferment. It was suggested that an investigation of the character of this "ferment" be made.

In this investigation the effect of age and of heat on the ferment in different lots of *Cascara sagrada* has been made. It has been found that the ferment in *Cascara sagrada* readily hydrolyzes starch. This ferment has amylase-like activity, but it is not destroyed by aging nor by moist heat at 100° in five minutes.

EXPERIMENTAL

Source of Cascara sagrada Used in This Investigation.—The *Cascara sagrada* used in this investigation came in four lots: three of them from the State of Washington,[1] and one from Massachusetts.[2] The latter was collected in Massachusetts in order to obtain a convenient supply for quick delivery. Samples of *Cascara sagrada* collected in 1949, 1950, and 1951 in the State of Massachusetts were not available.[3]

Procedure A

Preliminary Investigation.—Preliminary investigation during 1950–1951 showed that potato starch was readily hydrolyzed by the ferment in *Cascara sagrada*. All the experiments reported below, in which starch was used as the substrate, were run on the same batch of potato starch. All the experimental comparative observations to determine whether the ferment was destroyed by being aged were made in the same year, 1952.

Pulverization.—The bark could be easily ground to a suitable powder by an electric drug grinder. The powder was not sifted because all portions of the powder gave approximately the same results when they were assayed.

Drying.—The powdered bark was dried in one of two ways: *Method I.*—The ground bark was air-dried at room temperature for seven days, during which the freshly harvested bark lost approximately 37% of its weight. During the same interval loss from lots which had been in storage for a year or more was negligible.

Method II.—The ground bark was oven-dried at 100° for seven days. The freshly harvested bark lost about 53% of its weight in this time. The lots which had been in storage for a year or more lost approximately 8% of their weight during oven-drying.

Preparation of Filtrate for Analysis.—Filtrates were prepared from the air-dried and from the oven-dried samples in one of two ways: *Method I.*—One gram from each lot of *Cascara sagrada* was macerated in 50 cc. of water for one hour. The macerated material was then filtered through Whatman No. 1 filter paper, and the filtrate was assayed for amylase-like activity.

Method II.—One gram from each lot of *Cascara sagrada* was added to 50 cc. of water and was brought to boiling. It was allowed to simmer for five minutes. The source of heat was then removed and the mixture was allowed to macerate for one hour. It was then filtered as described above and the filtrate was assayed for amylase-like activity.

This procedure gave four different filtrates for each lot of *Cascara sagrada:* (*a*) air-dried at room temperature and macerated at room temperature, (*b*) air-dried at room temperature and brought to boiling before maceration, (*c*) oven-dried at 100° and macerated at room temperature, and (*d*) oven-dried at 100° and brought to boiling before maceration.

Preparation of Digestion Mixture and the Control.—In the digestion mixture the following ingredients were used: *Cascara sagrada* filtrate 5 cc.; physiological salt solution, 2 cc.; phosphate buffer solution, *p*H 7, 2 cc.; 1% solution of potato starch, freshly prepared, 5 cc.; and distilled water 6 cc.

In the control the *Cascara sagrada* filtrate was omitted, but 11 cc. of distilled water was used to bring the volume to 20 cc.

Preparation and Use of Special Iodine Test Solution.—One cc. of 0.1 *N* iodine test solution was diluted with distilled water to make 125 cc.; 1 cc. of this special iodine test solution was placed in the depression of a white spot plate. The end point was considered to be reached when one drop of the digestion mixture added to 1 cc. of this test solution was free of a blue color.

The digestion mixture and the control were each placed in 125-cc. Erlenmeyer flasks. The flasks were placed in a constant temperature water bath at 40° ± 1°. Each flask contained a dropper pipet. One drop from each mixture was tested at one-minute intervals until no blue color was obtained. A comparison of the hydrolytic activity of the amylase-like activity of *Cascara sagrada* collected at different times and prepared in four different ways may be seen in Table I.

The properties of the hydrolytic agent in the different lots of *Cascara sagrada* when assayed by this method may be listed as follows: (*a*) it is stable to heat; (*b*) it is not lost on storage; (*c*) it is partially inactivated when heated at 100°; and (*d*) the partially inactivated hydrolytic agent is reactivated by moist heat.

The hydrolytic agent is not a typical enzyme if one accepts the proposition that an enzyme is readily destroyed by moist heat (3). However, lysozyme is often classified as an enzyme and it is very stable to moist heat (4).

Procedure B

In this procedure photometric methods were used in an attempt to compare activity. These methods were not successful because of the deep colors of the filtrates from the *Cascara sagrada* samples. Dilutions made to overcome this interference simul-

[1] The lots of *Cascara sagrada* collected in 1949, 1950, and 1951 were supplied through the courtesy of Parke, Davis and Company.

[2] The lot of *Cascara sagrada* collected in 1952 was obtained from the medicinal plant garden of the Massachusetts College of Pharmacy.

[3] All of the samples of *Cascara sagrada* used in this investigation were authenticated by Professor Heber W. Youngken and Professor Maynard W. Quimby of the Massachusetts College of Pharmacy.

TABLE I.—THE EFFECT OF STORAGE AND OF HEAT ON THE HYDROLYTIC AGENT IN DIFFERENT LOTS OF CASCARA SAGRADA

Year Collected	Age of Bark	Time in Minutes for the Complete Hydrolysis of Starch: Air-Dried at RT for Seven Days — Macer. at RT	Air-Dried at RT for Seven Days — Boiled before Macer.	Oven-Dried at 100° for Seven Days — Macer. at RT	Oven-Dried at 100° for Seven Days — Boiled before Macer.
1952	Fresh	<1	<1	<1	<1
1951	1 yr.	<1	<1	7	<1
1950	2 yr.	<1	<1	36	<1
1949	3 yr.	...	<1	90	<1
Control		...	...	...	...

taneously reduced the active principle content to a point too minute for measurement by photometric means.

Procedure C

A Study of the Hydrolytic Activity of the Different Lots of Cascara sagrada by Serial Dilutions of the Filtrate.—It was obvious from the previous assay that air-drying at room temperature for seven days was satisfactory for the purposes of this investigation. It was equally obvious that the dried bark showed its maximum hydrolytic activity after the aqueous mixture was brought to boiling before maceration. Hence in the following studies the bark was first air-dried at room temperature and the aqueous mixture was brought to a boil for five minutes and was allowed to macerate for one hour before being filtered.

Preparation of Filtrate and of Serial Dilutions.—An amount of 5.562 Gm. of powdered air-dried *Cascara sagrada* was added to 50 cc. of distilled water. This mixture was brought to a boil and was allowed to simmer for five minutes. The mixture was macerated at room temperature for two days. The mixture was centrifuged, then filtered through folded gauze. The filtrate obtained from each lot of *Cascara sagrada* was diluted with water to obtain ten different concentrations for each lot. Then 5 cc. of each dilution was assayed as outlined in *Procedure A*. The concentrations of *Cascara sagrada* represented in 5 cc. of the diluted filtrate used in each assay are shown in Table II.

Composition of Digestion Mixture Containing Diluted Filtrate.—Five cubic centimeters of the diluted filtrate was added to a digestion mixture previously prepared as shown under *Procedure A*. The mixture was shaken for about five seconds. Then one drop of this mixture was added to an iodine test solution. In this analysis there was no color produced in the more concentrated solutions, but a blue color was obtained in the more highly diluted solutions of each lot of *Cascara sagrada*.

TABLE II.—CONCENTRATIONS OF CASCARA SAGRADA REPRESENTED IN DILUTED FILTRATE USED IN THE ANALYSIS

Dilution Number	Filtrate Used, cc.	Distilled Water Added, cc.	Total Volume	*Cascara sagrada* in 5 cc. Used in Analysis, Gm.
1	9	1	10	0.500
2	8	2	10	0.444
3	7	3	10	0.389
4	6	4	10	0.333
5	5	5	10	0.277
6	4	6	10	0.222
7	3.5	6.5	10	0.195
8	3	7	10	0.167
9	2	8	10	0.111
10	1	9	10	0.056

It was shown in *Procedure A* that the lots of *Cascara sagrada* collected in 1951 and in 1952 contained a more active hydrolytic agent than the 1949 and 1950 lots when the different lots were oven-dried at 100° for seven days and the bark was assayed after maceration without the use of moist heat. (See Table I.) The results obtained by *Procedure C* not only confirm but also extend the interpretations that can be made from the findings recorded in Table I. In *Procedure A* there was no difference in the hydrolytic activity in the air-dried lots of *Cascara sagrada* that were boiled before maceration. In this dilution method it is shown that the 1951 lot of the drug contained more of the hydrolytic agent than did either the 1949 or the 1950 air-dried lots.

Procedure D

Titration Method.—To determine the amount of *Cascara sagrada* required to hydrolyze a definite amount of starch in a given period of time a titration method was tried.

Preparation of Filtrate.—Five grams of each lot of *Cascara sagrada* was added to 50 cc. of distilled water. This mixture was brought to a boil and allowed to macerate for two days at room temperature. Following filtration, as outlined before, the filtrate of each lot was assayed.

Preliminary Assay.—The approximate number of cc. of each filtrate required to hydrolyze completely the 1% starch solution in the digestion mixture at room temperature was determined.

Assay Procedure.—Eight determinations were made on each filtrate. For the 1949 lot 2.3 cc. of filtrate was added to the digestion mixture rapidly from a 10-cc. buret. Then one drop at a time was added; and after each drop of filtrate was added to the digestion mixture, one drop was removed and added to an iodine test solution. For the 1950 lot 2.2 cc. of the filtrate was rapidly added; and then one drop at a time was removed as above. For the 1951 lot 1.4 cc. was the starting amount. The results of this method of analysis are shown in Table IV.

The results obtained by this method of analysis confirm the previous findings shown in Tables I and III. The 1951 lot had greater activity than

TABLE III.—HYDROLYTIC ACTIVITY OF DILUTED FILTRATE OF DIFFERENT LOTS OF CASCARA SAGRADA

Dilution Number	Year *Cascara sagrada* Was Collected 1949	1950	1951
1	No color	No color	No color
2	No color	No color	No color
3	No color	No color	No color
4	No color	No color	No color
5	No color	No color	No color
6	No color	No color	No color
7	End point	End point	No color
8	Deep blue	Deep blue	No color
9	Deep blue	Deep blue	End point
10	Deep blue	Deep blue	Deep blue
Control	Deep blue	Deep blue	Deep blue

either the 1949 or the 1950 lot. The results shown in Table IV have been submitted to statistical evaluation and found to be significant. The analysis of variance shows a real variation between lots. The criterion of likelihood shows a real uniformity within lots (5).

ISOLATION OF THE HYDROLYTIC AGENT IN CASCARA SAGRADA

Procedures to isolate the hydrolytic agent were carried out on three different lots of *Cascara sagrada*.

Procedure.—About 45 grams of the air-dried powder of *Cascara sagrada* was added to 200 cc. of distilled water, brought to a boil, and allowed to simmer for five minutes. Then the mixture was allowed to macerate at room temperature for four days.

The macerated mixture was filtered through folded gauze. This yielded approximately 100 cc. of filtrate. The filtrate was heated to 70°, and 80 cc. of alcohol was added. A slight precipitate formed, but was easily separated by filtration. The precipitate was discarded since it possessed only slight hydrolytic activity.

A dark gray precipitate was obtained when 200 cc. of alcohol was added to the clear filtrate, at room temperature. This precipitate was removed by centrifuging at 1800 r. p. m. The supernatant fluid was filtered through folded gauze. The precipitate showed only slight hydrolytic activity and was discarded.

TABLE IV.—ASSAY RESULTS OF DIFFERENT LOTS OF CASCARA SAGRADA BY TITRATION METHOD OF ANALYSIS OF FILTRATE

Trial Number	Filtrate of Each Lot of *Cascara sagrada* for Complete Hydrolysis of Starch, cc. 1949	1950	1951
1	2.35	2.95	2.05
2	2.40	2.20	1.85
3	2.75	2.50	1.80
4	2.90	2.25	1.60
5	2.45	2.55	1.70
6	2.50	2.85	1.55
7	2.90	2.95	2.00
8	2.80	2.55	1.45
Lot mean ($\bar{X}$)	2.63	2.60	1.75
Lot range (R)	0.55	0.75	0.60

The clear filtrate was then heated on a water bath until practically alcohol-free. Then distilled water was added to bring the volume to 100 cc. To this mixture an equal volume of a saturated solution of ammonium sulfate was added. This mixture was stored for two days, at room temperature, in an evaporating dish (toluene was added as a preservative). A dark, oily-like precipitate formed which was removed by filtration. This precipitate showed only slight hydrolytic activity and was discarded.

The filtrate was diluted with distilled water to make a volume of 100 cc. This dilution was divided into four 25-cc. portions and each was subjected to dialysis. After two days of dialysis all of the ammonium sulfate was washed out of the mixture.

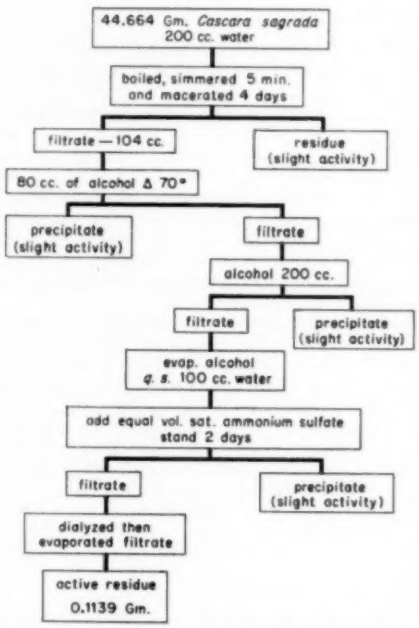

Fig. 1.—Flow chart for isolation of hydrolytic agent from *Cascara sagrada*, 1951 lot.

The solutions in the dialyzing tubes were then evaporated to dryness on a water bath. A black, shiny, glass-like substance was obtained. It had a melting point of about 220°. The precipitates from the different lots showed approximately the same degree of hydrolytic activity. The hydrolytic activity of this residue is approximately ten times greater than that of the lot of *Cascara sagrada* from which it was obtained. An example of the steps involved in this procedure is shown in Fig. 1.

SUMMARY

1. The hydrolytic agent in *Cascara sagrada* has the following properties: (*a*) it readily hydrolyzes starch; (*b*) it is not lost on aging; (*c*)

it is not destroyed by moist heat at 100°; (*d*) it is partially inactivated by dry heat at 100° after a period of seven days of heating but is reactivated by boiling water; and (*e*) it is soluble in water.

2. A method of analysis for the hydrolytic agent in different lots of *Cascara sagrada* has been worked out and evaluated as follows: (*a*) The results have been treated statistically and have been found to be reliable. (*b*) The 1951 lot had greater activity than either the 1950 or the 1949 lot. (*c*) The 1950 and 1949 lots showed no significant difference. (*d*) There was no significant difference in the results of the assays of the same lot.

3. The hydrolytic agent has been isolated as an impure residue. This residue is a black, shiny, glass-like, sticky substance with a melting point of about 220°.

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A Note on the Flavoring of Methionine and Choline Combinations*

By HERBERT M. GROSS

MEDICINAL PRODUCTS containing methionine and the choline salts in combination with inositol, thiamine, riboflavin, and liver are abundant in the physician's medicamentorum. These formulations provide the lipotropic principles necessary to reverse fatty liver infiltration and prevent hepatic destruction. While the individual characteristics of these substances pose a flavoring problem, the combination of these ingredients is nauseating. The necessity for an appealing flavor to disguise this objectionable taste is obvious.

EXPERIMENTAL

A formulation containing 400 mg. choline dihydrogen citrate, 100 mg. methionine, 10 mg. inositol, 2 mg. thiamine hydrochloride, 2 mg. riboflavin, 10 mg. niacinamide, 5 μg. cyanocobalamin, and 470 mg. liver extract per 4 cc. (one teaspoonful) was prepared. This aqueous vehicle, exemplifying several commercial preparations, contained 10% glycerin as solubilizer and 0.1% methyl *para*-hydroxybenzoate as a preservative. After preliminary tasting of the raw formulation, it was apparent that some sweetening agent would be necessary. Cyclamate sodium was used in a 0.01% concentration as an adjunct to eliminate the bitterness of saccharin and the use of sucrose which reportedly is incompatible with cyanocobalamin (1). The glycerin in the formula also contributed to the sweetness.

The raw formulation had both an offensive, putrid odor and a nauseating, bitter, fishy taste. The addition of 3% polysorbate 80 to the formulation had a marked influence on the reduction of the nauseating odor. This substance contributed a soapy factor to the taste, but the therapeutic indications of lipotropic activity (2) were felt to overshadow this disadvantage.

Several manufacturers of flavoring materials were contacted and their suggested samples were combined with the lipotropic solution at a 1% concentration in a comparative study of their odor- and taste-masking properties.

Hamann (3) states that flavor has three components: odor, taste, and consistency, and he reports that consistency has a direct influence on palatability. The effectiveness of flavor enhancement by viscosity functions on the principle that the viscid disguising flavor, due to the tenacity caused by viscosity, is diminished at a slower rate than the solution time of the bitter principle. The viscosity of this preparation was adjusted by introducing 0.5% carboxymethylcellulose (medium viscosity).

A panel comprised of 18 adults, male and female, indicated the tabulated preferences. Each subject tested the samples on two occasions. The necessity for a trained flavor panel in testing this formulation was diminished by its inherent unpleasantness. Little difficulty was encountered with the nauseous obviousness of odor and taste, and once instructed in the method of determining taste and aftertaste, reproducible ratings were possible with an inexperienced panel. Odor coverage was first tested, then a small sample was tasted by swishing it about in the mouth and finally spitting out the sample. Both immediate and aftertastes were considered in this method outlined by Crocker (4). The mouth was rinsed between samples with lukewarm water. Since the formulation had a brown color, no artificial colors were added to the samples so as to limit any psychological effect of color on taste.

* Received December 7, 1953, from the Department of Pharmaceutical Development, Commercial Solvents Corporation, Terre Haute, Ind.

TABLE I.—TASTE PANEL PREFERENCES ON FLAVORS USED TO DISGUISE CHOLINE, METHIONINE, AND LIVER FORMULATIONS

Flavor Sample	Average Panel Rating per Flavor Characteristic			
	Odor	Taste	Consistency	Mean Flavor Rating
Imitation raspberry[b]	3.4[a]	1.2	1.6	2.1
Imitation cherry[d]	2.0	2.0	1.6	1.9
Wild cherry, imitation[b]	2.3	2.1	1.6	2.0
Grenadine, imitation (conc.)[e]	3.1	1.0	2.8	2.3
Orange concentrate[b]	1.1	3.1	2.0	2.1
Sesquiterpeneless orange oil[c]	3.4	3.6	2.0	3.0
Lemon concentrate, fivefold[b]	1.4	2.7	1.8	2.0
Sesquiterpeneless lemon oil[b]	3.8	3.2	1.8	2.9
Cocoa syrup, U. S. P.	1.2	2.1	1.8	1.7
Imitation coconut custard[d]	3.2	4.3	3.5	3.7
Imitation butter caramel[c]	4.0	4.8	4.2	4.3

[a] Numbers based on 18 individual duplicate ratings of: 1—nauseous; 2—bad; 3—fair; 4—good; 5—excellent.
[b] Fritzsche Brothers, Inc., New York, N. Y.
[c] Magnus, Mabee, and Raynard, Inc., New York, N. Y.
[d] Flavor E-515-S, Van Ameringen-Haebler, Inc., Elizabeth, N. J.
[e] Flavor #3281, Van Ameringen-Haebler, Inc., Elizabeth, N. J.

DISCUSSION

Imitation butter caramel flavor was indicated as having the most suitable characteristics to mask the disagreeable taste and odor of methionine, choline, and liver mixtures. The intensity of this flavor matches the intensity of the taste of the disagreeable constituents and thus effectively masks them.

An interesting sidelight with respect to this flavor was that coffee drinkers thought the sample tested had a pleasant coffee flavor, while noncoffee drinkers thought the sample had the agreeable taste of maple syrup. This flavor was imparted by the flavoring agent. Coffee extractives, vanillin, and other synthetic aldehydes, diacetyl and other ketones, ethylbutyrate, glycerin, ethyl alcohol, water, and caramel color comprise the mixture named "imitation butter caramel flavor."

Imitation coconut custard flavor had good characteristics for immediate taste and odor coverage, but it did not mask the disagreeable aftertaste.

SUMMARY

Of the flavors tested, imitation butter caramel flavor has excellent characteristics for masking the disagreeable odor and taste of methionine, choline, and liver formulations.

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Book Notices

The Pharmacist in Retail Distribution. By A. HAMILTON CHUTE and ESTHER J. W. HALL. Hemphill's Book Stores, Austin, Texas, 1953. viii + 356 pp. 22 x 28 cm. Paper cover, ringbound. Price $6.

This book is intended as a text for the phase of the distribution of drugs by retail means. It is partly a revision of *Retailing by Pharmacists* by Chute (1941), to which has been added 12 new chapters, with the remainder substantially modernized, rearranged, and rewritten. The arrangement was modified to conform largely to the outline recommended by a committee as reported in *The Pharmaceutical Curriculum*. The text is intended primarily for use by students in the College of Pharmacy at the University of Texas, but students at other colleges would find it useful.

The book covers the many aspects of retail pharmacy and includes topics dealing with the pharmacist and the marketing process, the pharmacy among retail institutions, selecting a location for a pharmacy, type of pharmacy to select, purchasing and financing of the business, organization of the pharmacy, and operation of the pharmacy with various business policies and legal and ethical aspects of retail pharmacy. Some of the chapters contain photographs of drug store interiors showing layouts for the prescription department and other departments, such as proprietary, cosmetic, and the soda fountain, which may be helpful to the pharmacist in organizing his business.

The Chemistry of the Morphine Alkaloids. By K. W. BENTLEY. (British Publisher: Clarendon Press) Oxford University Press, New York, N. Y., 1954. 452 pp. 14.5 x 23.5 cm. Cloth. Price $8.

This volume is the first of a series of monographs on the Chemistry of Natural Products. It is an excellent reference work in a very specialized field, and is thoroughly up to date; it contains, indeed, considerable information which has not yet appeared in current journals. As the title indicates, there is no discussion of opium, of the "minor" (benzylisoquinoline) alkaloids, or of physiological action. It is a book for morphine chemists written by a morphine chemist; nevertheless, the extraordinary reactions and rearrangements exhibited by the versatile morphine-thebaine heterocyclic systems are such that no investigator in the field of nitrogen heterocycles can afford to neglect them.

In the foreword, Professor Robinson intimates that some significant contributions of English chemists have not been hitherto seen in proper perspective, particularly in the recent reviews by Manske and Holmes. With this viewpoint I cannot agree, and on the other side of the picture may be permitted to mention that no English chemist has ever credited Robert Pschorr [*Liebig's Annalen*, **373**, 64(1910)] with the reasoning which represents the key to the morphine-ring system, a structural deduction so lucid that one cannot today understand why Pschorr failed to elaborate it.

The present monograph, like others in the field, treats the individual phenanthrene alkaloids in separate chapters, and the most important groups of derivatives similarly, each chapter with its own bibliography (in all, twenty-eight sections, with an appendix of very recent work). The chemistry of sinomenine and hasubanonine is logically included, and because of the relatively inaccessible references (mostly Japanese) is most welcome.

The monograph is a straightforward account of the published literature, and is not burdened with speculative, if sometimes stimulating, discussions (although the section on Flavothebaone may come into this class). Typography is exceptional; two minor errors may be noted: p. 29, *gamma*-isomorphine is attributed to the hydrolysis of *alpha*-chlorocodide, where *alpha*-chloromorphide is meant, and on p. 80, line 2, "demethylation" should read "methylation." The descriptive sections, although concentrated in tabular form, seem to be complete in themselves, and do not necessitate reference to the out-of-print *Chemistry of the Opium Alkaloids* of Small and Lutz. The index is adequate, but might well have included the trade names of such common drugs as Dicodid, Dilaudid, Acedicon, Eukodal, and Nalline or their international (United Nations) equivalents.

If any criticism can be offered it would be on nomenclature. Some chemists who have devoted most of their lives to this fascinating group of alkaloids have a certain reverence for the old pioneers in the field, Knorr, Pschorr, Vongerichten, and Freund, and the terms in which their laborious progress was expressed. There seems to be little justification for change of the historic and well-established nomenclature of the methylmorphimethines (Hesse, 1883) to codeimethines. Add to confusion the statement on p. 101, that methylmorphimethine will be applied to nuclear methylated morphines, but on p. 110 precisely these nuclear methylated morphine degradation products are designated as 6-methyl codeimethines. In the thebainone-dihydrothebainone and dihydrothebaine series the situation becomes even more complicated. Greek-letter prefixes, suffixes, and Greek-Roman are mixed to the point of distraction. Every research chemist in steroids, alkaloids, or another complex field, encounters the problem of assigning a trivial designation until such time as final structure elucidation (location of unsaturation, configuration of asymmetric centers) permits adequate naming. The thebaine-thebainone nomenclature which has slowly evolved during the last 25 years is admittedly not satisfactory. The present proposals do not result in improvement. Dihydrothebaine-ϕ (ϕ means phenyl to most chemists and is so used on p. 268 and elsewhere) is naturally to be associated with the unrelated phenyldihydrothebaine. In the ketonic series then appear (p. 213) thebainone-A, β-thebainone-A, thebainone-C, thebainone-β (evidently a misprint), and thebainone-B until the mind reels. Thebainone-C is, in fact, no longer a thebainone, but a methine type. Since structures in this series are apparently fairly well established, one can only regret that the proposal of the Editor of the Journal of the Chemical Society for naming (*J. Chem. Soc.*, **1952**, 969, footnote) was not adopted.

Disregarding these minor criticisms, the volume is a scholarly work, representing an enormous amount of labor, much of which will be spared those who have occasion to study or investigate further in this field, which is by no means exhausted.

LYNDON F. SMALL
National Institutes of Health

Fractionnements par Solvants. By MAURICE VIGNERON. Vigot Frères, Editeurs, Paris, 1954. 152 pp. Illustr. 16 x 24.5 cm. Price *Fr.* 1,600.

This book contains, in a revised and enlarged form, four lectures given by the author before the Institute of Industrial Pharmacy of Paris. It discusses the solvent extraction of liquids from solids and liquids from liquids, fractionation by chromatography, and ion exchange processes. The approach is not theoretical or bibliographical, but practical and based on the pharmaceutical and industrial experiences of the author. At the same time, each chapter is documented by a useful "summary bibliography." Numerous illustrations of apparatus, tables, and charts enhance the value of the work so that it may be used successfully even by those whose knowledge of French goes back to some dimly remembered high school course.

Handbook of Cosmetic Materials—Their Properties, Uses, and Toxic and Dermatologic Actions. By LEON A. GREENBERG and DAVID LESTER. Interscience Publishers, Inc., New York, 1954. xii + 455 pp. 16.5 x 23.5 cm. Price $12.50.

This handbook will be found useful by members of the cosmetic and allied industries and also by chemists, physicians, dermatologists, and allergists. The book is based on a collaborative effort of the cosmetic industry, initiated by the Toilet Goods Asso-

ciation, and includes approximately 1,000 materials which were selected in response to a questionnaire sent to cosmetic manufacturers.

The following data on the materials are given: formula, properties, uses, toxic action, and dermatologic action. The compounds are listed alphabetically; the information on each is given under its more widely used name and numerous cross references list trade names, synonyms, and scientific names. References to the literature are cited for many of the compounds and a bibliography of 2,500 items is provided which consists of all works actually referred to in the text plus other collateral information.

A chapter on the skin is also included in this handbook, thus affording information helpful in understanding the physiological aspects of the dermatological action of the compounds.

BOOKS RECEIVED

Vegetable Tanning Materials. By F. N. HOWES. Butterworth's Scientific Publications, Ltd., London, 1953. (American Distributor: The Chronica Botanica Co., Waltham, Mass.) 325 pp. Illustr. 14.5 x 22 cm. Price $5.50.

Encyclopedia of Chemical Technology. Vol. 12 (Sabadine to Stilbestrol.) RAYMOND E. KIRK and DONALD F. OTHMER, Editors. The Interscience Encyclopedia, Inc., New York, 1954. xvi + 955 pp. Illustr. 20 x 27 cm. Price $30 (single copy), $25 (subscription price).

Antibiotika-Fibel—Indikation und Anwendung der Chemotherapeutika und Antibiotika. By ALBERT M. WALTER and LUDWIG HEILMEYER. Georg Thieme Verlag, Stuttgart, 1954. (In the U. S. and Canada: Intercontinental Medical Book Corporation, New York.) 813 pp. 16 x 21 cm. Price *DM.* 79 ($18.80).

Industrial Fermentations. By LELAND A. UNDERKOFLER and RICHARD J. HICKEY. Vol. I. Chemical Publishing Co., Inc., New York, 1954. x and 565 pp. Illustr. 14.5 x 22.5 cm. Price $12.

Annals of the New York Academy of Sciences. Vol. 59, Art. 1, pp. 1–140. ROY WALDO MINER, Editor. Reserpine (Serpasil) and Other Alkaloids of *Rauwolfia Serpentia:* Chemistry, Pharmacology, and Clinical Applications. The New York Academy of Sciences, New York, 1954. 15.5 x 23 cm. 140 pp. Price $3.

Compounds with Condensed Thiopene Rings. By H. D. HARTOUGH and S. L. MEISEL. Seventh volume in the series *The Chemistry of Heterocyclic Compounds.* Interscience Publishers Inc., New York, 1954. 515 pp. 16.5 x 24 cm. Price $16.50; Subscription Price $15.

Newer Concepts of the Causes and Treatment of Diabetes Mellitus. Proceedings of the Symposium on Diabetes Sponsored by the New York Diabetes Association. The National Vitamin Foundation, Inc., 1954. 181 pp. 15.5 x 23 cm. Price $2.50.

Wine as Food and Medicine. By Salvatore P. Lucia. The Blakiston Company, Inc., New York, 1954. 149 pp. 14 x 21.5 cm. Price $3.

The Science Book of Wonder Drugs. By DONALD G. COOLEY. Franklin Watts, Inc., New York, 1954. 247 pp. 15 x 20 cm. Price $2.95.

Textbook of Organic Medicinal and Pharmaceutical Chemistry. 2nd ed. CHARLES O. WILSON and OLE GISVOLD, Editors. J. B. Lippincott Co., Philadelphia, 1954. 807 pp. 19 x 26.5 cm. Price $10.

Kurzes Lehrbuch der Pharmazeutischen Chemie, auch zum Gebrauch fuer Mediziner. (Textbook of Pharmaceutical Chemistry, also for the use of physicians.) By K. BODENDORF. 4th rev. ed. Springer Verlag, Berlin, 1954. 482 pp. 18 x 25.5 cm. Price *DM.* 31.50.

The Surgical Appliance Technician's Handbook. By HOWARD L. BOYLAND. Surgical Appliance Industries, Inc., Cincinnati, Ohio, 1954. 459 pp. Illustr. 16.5 x 24 cm. Price $10.

Arrest of Bleeding—Physiology, Pharmacology, Pathology. By JACQUES ROSKAM. Charles C Thomas, Publisher, Springfield, Ill., 1954. 71 pp. 14.5 x 22 cm. Paper cover. Price $2.75.

Les Détersifs en Hygiène et Cosmétique. By W. KOPACZEWSKI. Masson & Cie, Paris, 1954. 143 pp. 16 x 25 cm. Paper bound, uncut. Price *Fr.* 1,800.

Histopathologic Technic and Practical Histochemistry. By R. D. LILLIE. The Blakiston Co., New York, 1954. 501 pp. 16.5 x 24.5 cm. Price $7.50.

Chemotherapy of Infections. By H. O. J. COLLIER. John Wiley & Sons, Inc., New York, 1954. xvi + 248 pp. 14.5 x 19.5 cm. Price $4.

Statistical Analysis in Chemistry and the Chemical Industry. By CARL A. BENNETT and NORMAN L. FRANKLIN. (Sponsored by the Committee on Applied Mathematical Statistics, The National Research Council.) John Wiley & Sons, Inc., New York (Chapman & Hall, Ltd., London), 1954. xvi + 724 pp. 16 x 23.7 cm. Price $8.

Advances in Enzymology and Related Subjects of Biochemistry. Vol. XV. F. F. NORD, Editor. Interscience Publishers, Inc., New York, 1954. 547 pp. 16.5 x 24 cm. Price $11.

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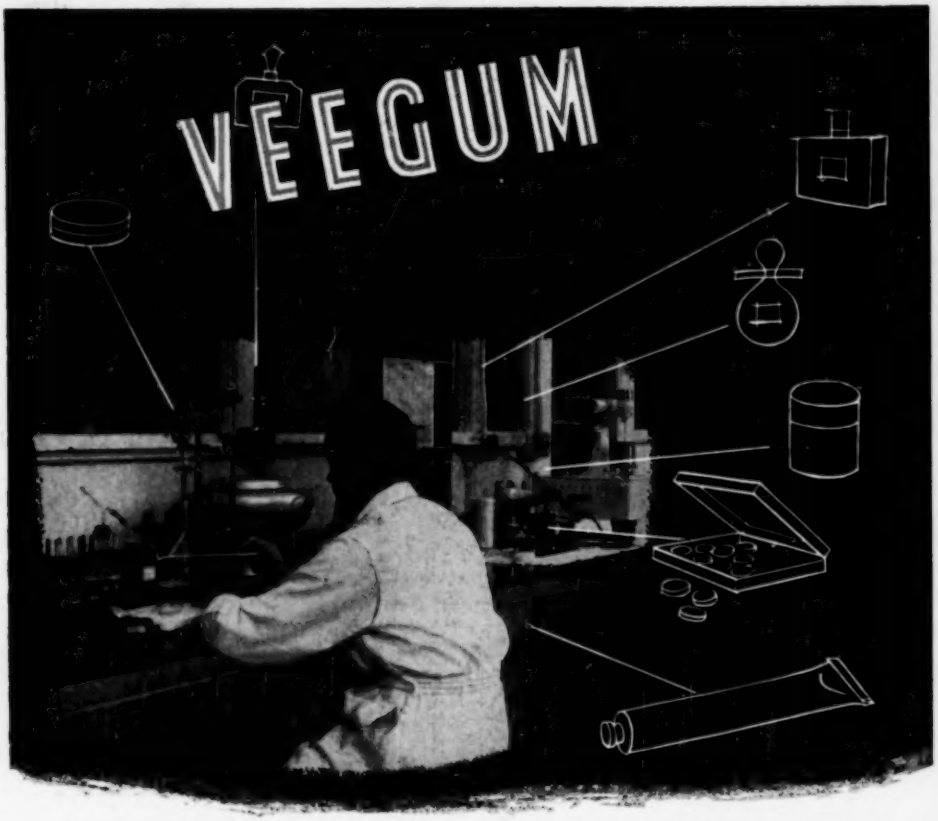

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